The Pennsylvania State University
The Graduate School
College of Medicine

STRESS-INDUCED GLUCOCORTICOID IMPAIR DENDRITIC CELL FUNCTION,
COMPROMISING CD8+ T CELL RESPONSES TO HERPES SIMPLEX VIRUS

A Dissertation in
Immunology and Infectious Diseases

by
Michael D. Elftman

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2009
The dissertation of Michael D. Elftman was reviewed and approved* by the following:

Robert H. Bonneau  
Professor of Microbiology and Immunology  
Dissertation Co-advisor  
Co-chair of Committee

Mary E. Truckenmiller  
Assistant Professor of Microbiology and Immunology  
Dissertation Co-advisor  
Co-chair of Committee

Christopher C. Norbury  
Associate Professor of Microbiology and Immunology

Avery August  
Professor of Immunology

D. Channe Gowda  
Professor of Biochemistry and Molecular Biology

Neil D. Christensen  
Associate Professor of Pathology, and Microbiology and Immunology  
Co-chair of Intercollege Graduate Degree Program in Immunology and Infectious Diseases

*Signatures are on file in the Graduate School
ABSTRACT

Chronic psychological stress has long been known to be potently immunosuppressive. Although several studies have found that stress suppresses CD8\(^+\) T cell-mediated immunity to herpes simplex virus (HSV), the underlying mechanisms of this suppression and the specific cellular targets of neuroendocrine hormones are unknown.

In order to generate an antigen-specific CD8\(^+\) T cell response, a protein must be processed into an 8-10 amino acid long antigenic peptide. These peptides are presented to antigen-specific CD8\(^+\) T cells on the appropriate MHC class I molecule by antigen-presenting cells (APC) that express the necessary costimulatory molecules. Interfering with any of these steps can result in weaker or slower CD8\(^+\) T cell responses, thus impairing the ability of the host to control the growth and spread of a pathogen.

Dendritic cells (DC) are the most potent APC and are critical in the initiation of CD8\(^+\) T cell responses. While neuroendocrine hormones, including stress-induced glucocorticoids (GC), have strong suppressive effects on T cell responses, it is not known whether these effects are mediated by direct effects on T cells themselves or occur indirectly via effects on the DC that are necessary for T cell activation.

The effects of stress-induced GC on DC function were investigated by treating murine DC with the rodent GC corticosterone (CORT) and determining the effects of CORT on the responses of DC to lipopolysaccharide (LPS). These studies found that CORT suppresses several components of the DC response to LPS, with effects on DC maturation, endocytosis, and cytokine production. The net result of the effects of CORT on DC is that DC are rendered less efficient for T cell priming.
These studies were extended in vivo to determine the effects of stress on DC function during an HSV infection. By using a model of restraint stress in which mice were subjected to stress during the earliest part of infection, we were able to determine the effects of stress on the first stages of the immune response and the consequences of these effects on the subsequent course of infection. In these studies, we found that DC from infected mice were impaired in their ability to stimulate CD8⁺ T cells ex vivo, while the CD8⁺ T cell response was impaired in vivo. These impairments were associated with increased viral loads, delayed viral clearance, and delayed resolution of viral lesions. The effects of stress on the CD8⁺ T cell response are mediated by CORT because they can be blocked using a GC receptor (GR) antagonist. Using a line of mice whose T cells do not express GR (GR-TKO), we were able to determine that CD8⁺ T cell responses are suppressed by the effects of stress-induced CORT on non-T cells. The identity of these “non-T cells” is likely to be DC, as they are impaired in stressed mice, critical for efficient T cell function, and susceptible to functional impairment by CORT.

The findings presented in this dissertation underscore the central role that DC have in the generation of T cell responses and highlight the importance of DC as a target of neuroendocrine hormones during stress-induced immunosuppression. Therapeutic strategies aimed at modulating T cell responses in stressed individuals must take into account the effects of neuroendocrine hormones on DC function to successfully overcome stress-induced immunosuppression.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xviii</td>
</tr>
<tr>
<td>CHAPTER I: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER II: LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>A. Immune Responses</td>
<td>5</td>
</tr>
<tr>
<td>1. Innate immunity</td>
<td>5</td>
</tr>
<tr>
<td>a. Pathogen recognition</td>
<td>5</td>
</tr>
<tr>
<td>b. Effectors of innate immunity</td>
<td>7</td>
</tr>
<tr>
<td>c. Innate antiviral immunity</td>
<td>8</td>
</tr>
<tr>
<td>b. Adaptive antiviral immunity</td>
<td>10</td>
</tr>
<tr>
<td>2. Antigen-presenting cells</td>
<td>12</td>
</tr>
<tr>
<td>a. Introduction to DC</td>
<td>12</td>
</tr>
<tr>
<td>b. DC ontogeny</td>
<td>13</td>
</tr>
<tr>
<td>c. Properties of DC</td>
<td>15</td>
</tr>
<tr>
<td>i. Immature DC</td>
<td>15</td>
</tr>
<tr>
<td>ii. DC maturation</td>
<td>16</td>
</tr>
<tr>
<td>iii. Antigen presentation</td>
<td>18</td>
</tr>
<tr>
<td>d. DC subsets</td>
<td>19</td>
</tr>
<tr>
<td>i. Conventional DC</td>
<td>19</td>
</tr>
<tr>
<td>i-a. Lymphoid-resident DC</td>
<td>19</td>
</tr>
</tbody>
</table>
i-b. Migratory DC.....................................................20

ii. Plasmacytoid DC (pDC).................................21

iii. Interferon-producing killer cells......................23

e. Role of DC in antiviral immunity.........................23

3. Adaptive immunity...........................................26
   a. Adaptive antiviral immunity.............................28

B. Neuroendocrine-immune interactions..................31
   1. The stress response........................................32
   2. Underlying mechanisms of stress/glucocorticoid (GC)-induced
      immunosuppression.........................................36
      a. General mechanisms of GC-induced immunosuppression.....37
      b. Effects of stress/GC on antiviral immunity..................39
         i. Effects of stress on CD8$^+$ T cell function – emphasis on
            herpes simplex virus (HSV)............................40
         ii. Effects of GC on DC function..........................42

CHAPTER III: CORTICOSTERONE IMPAIRS DENDRITIC CELL MATURATION
   AND FUNCTION................................................44

A. Introduction....................................................44

B. Materials and Methods.......................................46
   1. Mice............................................................46
   2. Generation of bone marrow-derived DC (BMDC)..............46
   3. Corticosterone (CORT)/lipopolysaccharide (LPS) treatment....46
   4. GC receptor (GR) antagonist treatment........................47
5. Analysis of protein expression by flow cytometry .........................47
6. Detection of apoptosis .................................................................48
7. Real-time polymerase chain reaction (PCR) ....................................48
8. Fluorescence microscopy .................................................................49
9. Antigen uptake ..............................................................................50
10. In vivo T cell priming .................................................................50
11. Intracellular cytokine staining ......................................................51
12. Degranulation assay ....................................................................51
13. Statistical analyses ......................................................................52

C. Results ..........................................................................................53

1. Generation of BMDC .................................................................53
2. CORT impairs LPS-induced upregulation of maturation-associated markers .................................................................53
3. DC maturation is impaired by physiological concentrations of CORT. 56
4. Effects of CORT are mediated through the GR and are not due to apoptosis .................................................................56
5. CORT impairs transcription of B7.1 and B7.2 and causes intracellular retention of MHC class II .................................................................59
6. CORT prevents LPS-induced downregulation of endocytosis ........65
7. CORT impairs cytokine production by DC ....................................70
8. CORT renders DC inefficient at priming CD8⁺ T cell responses ......70

D. Discussion ..................................................................................78
CHAPTER IV: CHRONIC STRESS IMPAIRS ANTIVIRAL CD8⁺ T CELL RESPONSES VIA EFFECTS ON DENDRITIC CELLS RESULTING IN INCREASED VIRAL PATHOGENESIS ..............................84

A. Introduction ..............................................................................................................84

B. Materials and Methods ..........................................................................................87

1. Mice .........................................................................................................................87

2. Restraint stress .........................................................................................................87

3. HSV infection ...........................................................................................................88

4. Exogenous CORT administration ..........................................................................88

5. GR antagonist administration ................................................................................88

6. HSV lesion scoring ..................................................................................................88

7. Quantification of infectious virus ............................................................................89

8. Quantitative PCR .....................................................................................................89

9. Western blotting ......................................................................................................89

10. Detection of antigen-specific CD8⁺ T cells ............................................................90

11. Intracellular cytokine staining ...............................................................................90

12. Degranulation assay ...............................................................................................91

13. Proliferation assay ..................................................................................................91

14. Statistical analyses ..................................................................................................92

C. Results ......................................................................................................................93

1. HSV pathology is exacerbated by stress applied early in infection.....93

2. Stress terminated early after infection is sufficient to impair HSV-
specific CD8⁺ T cell responses in the popliteal lymph node (PLN).....96
3. Stress impairs antiviral CD8⁺ T cell responses via the GR.............101

4. Stress-induced impairment of CD8⁺ T cell responses is not due to direct effects of CORT on the T cells.........................................................106

5. Impaired control of viral replication is not due to direct effects of stress-induced CORT on T cells.........................................................109

6. DC function is impaired by stress in vivo..........................................112

D. Discussion..........................................................................................116

CHAPTER V: ADDITIONAL FINDINGS.....................................................120

A. Stress does not impair DC maturation in PLN during HSV infection in vivo.................................................................120

B. CORT treatment does not impair macropinocytosis by DC............124

C. CD8α⁺ DC and pDC in the PLN are preferentially susceptible to stress-induced CORT.................................................................128

D. Naltrexone does not restore splenic cellularity in C57BL/6 and Balb/c mice.................................................................................132

E. HSV-infected mice subjected to stress in the early stages of infection have less viral DNA in dorsal root ganglia.................................................135

CHAPTER VI: DISCUSSION.......................................................................138

A. Implications for a model of CORT-induced impairment of DC function.....139

1. What accounts for the discrepancy between in vitro effects of CORT and in vivo effects of stress on DC maturation?.........................140

a. CORT does not equal stress..............................................................140

b. LPS does not equal HSV.................................................................141
c. BMDC are not equivalent to DC found in vivo………………..142
d. In vivo studies may not have been able to detect impairment of
DC maturation…………………………………………………………..142

2. What are the cellular targets for stress-induced CORT?…………….143
3. Are the increased viral load and delayed viral clearance and resolution
of lesions in stress mice due to impaired CD8+ T cell responses?…..146

B. Unanswered questions about the biology of stress-induced
immunosuppression…………………………………………………………..148

1. What are the mechanisms by which CORT affects DC?…………….148
2. What is the mechanism that underlies the decrease in cellularity of
secondary lymphoid tissues during psychological stress?…………..150

C. Conclusions………………………………………………………………….152

REFERENCES…………………………………………………………………………...157
LIST OF FIGURES

1. GR-binding molecules........................................................................................................34
2. Effects of CORT/LPS on DC maturation-associated marker surface expression........54
3. CORT acts at concentrations that saturate the GR and is blocked by pre-treatment
   with GR antagonist...........................................................................................................57
4. CORT does not induce apoptosis or downregulate TLR-4 on DC............................60
5. Effects of CORT/LPS on protein and mRNA expression of DC maturation-
   associated markers..........................................................................................................62
6. Effects of CORT/LPS on cellular localization of MHC class II...............................66
7. Effect of CORT/LPS on endocytosis of soluble protein............................................68
8. Effect of CORT/LPS on cytokine production by DC...............................................71
9. Effect of CORT on the ability of DC to prime CD8+ T cell responses in vivo.........74
10. Injection of empty DC alone does not induce a CD8+ T cell response...............76
11. Effect of CORT on CpG DNA or Poly (I:C)-induced DC maturation-associated
    marker surface expression.............................................................................................81
12. Restraint stress delays the resolution of HSV lesions on footpads.........................94
13. Stressed mice have higher HSV-1 load.........................................................................97
14. Stressed mice have more HSV-1 DNA in their PLN..................................................99
15. Stress impairs the CD8+ T cell response against HSV............................................102
16. The effects of stress on the CD8+ T cell response are mediated through the GR...104
17. CORT impairs CD8+ T cell responses independently of direct effects on T cells...107
18. Stress suppresses CD8+ T cell responses and limits control of viral replication
    independently of direct effects on T cells.......................................................................110
19. Stress impairs the ability of DC from HSV-infected mice to induce proliferation by antigen-specific CD8^+ T cells .......................................................... 114

20. Stress does not inhibit DC maturation in the PLN of HSV-infected mice .......... 122

21. CORT has no effect on macropinocytosis by BMDC .................................... 126

22. CD8α^+ and pDC in the PLN are preferentially susceptible to stress-induced depletion ........................................................................................................ 130

23. Naltrexone fails to restore splenic cellularity in stressed mice ....................... 133

24. Exposure to stress early in infection reduces the amount of HSV DNA in dorsal root ganglia .................................................................................................................. 136

25. A model for stress-induced suppression of immunity to HSV ......................... 154
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic-releasing hormone</td>
</tr>
<tr>
<td>AIM2</td>
<td>absent in melanoma-2</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate, succinimidyl ester</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLm</td>
<td>memory cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of interferon regulatory factor</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DN</td>
<td>double-negative</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
</tbody>
</table>
ER  endoplasmic reticulum
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
FLT3  fms-like tyrosine kinase 3
FLT3L  fms-like tyrosine kinase 3 ligand
gB  glycoprotein B
GC  glucocorticoid
GM-CSF  granulocyte-macrophage colony-stimulating factor
GR  glucocorticoid receptor
GRE  glucocorticoid response element
GR-T_{KO}  glucocorticoid receptor knockout in T cells
HPA  hypothalamic-pituitary-adrenal
HSV  herpes simplex virus
IFN  interferon
IL  interleukin
IκB  inhibitor of NFκB
IMDM  Iscove’s modified Dulbecco’s media
IRF  interferon regulatory factor
JAK  Janus kinase
LAMP  lysosome-associated membrane protein
LC  Langerhans cells
Lin  lineage
LN  lymph node
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LY</td>
<td>Lucifer yellow</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signaling</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-colony-stimulating factor</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MoDC</td>
<td>monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSH</td>
<td>melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation gene 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κ B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>OAS</td>
<td>oligoadenylate synthetase</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>pAPC</td>
<td>professional antigen-presenting cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>PLN</td>
<td>popliteal lymph node</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid-inducible gene-I</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SLC</td>
<td>secondary lymphoid tissue chemokine</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single-stranded ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T(_H)</td>
<td>helper T</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalitis virus</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>T(_{Reg})</td>
<td>regulatory T</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/IL-1 receptor-domain-containing adaptor protein inducing IFN-β</td>
</tr>
<tr>
<td>VEH</td>
<td>vehicle</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>-----</td>
<td>---------------</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Financial support for my studies at Penn State has been provided by the Huck Institute for Life Sciences, the National Institutes of Health (grants R01 AI065702 and 2 T32 CA60395-11), and Tobacco Settlement Funds from the Pennsylvania State Department of Health.

The completion of the studies presented in this dissertation could not have been achieved alone. There are several individuals whose guidance, friendship, and help have been invaluable over the past six years. First, I would like to thank my mentors, Dr. Robert Bonneau and Dr. Emmy Truckenmiller. Rob welcomed me into the lab and has been a tremendous source of encouragement and advice ever since. Emmy has always been there for me and has challenged me to think critically and to work to my fullest potential. I could never have asked for two better people to help me to think like the scientist I aspire to become.

To Jen Mellinger, I owe a great deal of thanks. She has been a reliable helper and true friend ever since she joined the lab. She has been my “second pair” of hands and eyes countless times, and could always be trusted to put her best efforts into every experiment. Her work ethic and organizational skills are inspiring, and I am grateful to have worked with her these past years.

Several other members and former members of the lab have provided friendship and advice, especially Dr. John Hunzeker, who always provided honest advice and reassurances that my graduate studies would not last forever, and Dr. Hassan Zahwa, whose unique perspective and sense of humor could always bring a smile to my face.
I would like to thank Dr. Richard Courtney for fostering a collaborative and supportive environment in the Department of Microbiology and Immunology and especially for the support I received on the departmental training grant. I am also indebted to the members of my graduate committee who have challenged me to consider all possible interpretations of my findings, Dr. Avery August, Dr. Channe Gowda, and especially Dr. Chris Norbury, who was often willing to provide advice about life as young scientist in academia in addition to generously sharing his thoughts on experiments and the field of immunology in general.

Several other people at the Penn State College of Medicine have been generous with advice and support, including Dr. Neil Christensen, director of the Immunology and Infectious Diseases program, Ray Scheetz and the staff of Central Lab Resources, Nate Schaeffer and Dr. Dave Stanford from the flow cytometry facility, the staff of the animal resource facility and Mel Epler, who was always willing to provide valuable technical advice on experiments.

There is no way that I could have been able to accomplish all that I have without the support and love of a wonderful family. I would like to thank my mom, Kathy, and my brother, Jeff, for their constant support and encouragement. I am also proud to have the inspiration of my late father, David, as the first “biologist” I have ever known. To my wife, Leah, I am eternally grateful. Her love, patience, and support were necessary for me to sustain my efforts in the lab these past years. She has given me a beautiful son, Samuel, a source of constant joy in my life.
Finally, I thank God, for His manifold blessings, especially patience and wisdom. I pray that I may continue to honor the Lord in all of my future endeavors and that His face may continue to shine upon me for the rest of my life.
CHAPTER I: INTRODUCTION

Psychological stress occurs when an individual experiences an actual or perceived threat to homeostasis, resulting in a physiological response. This response, known as the stress response, is aimed at reducing a threat and maintaining homeostasis by a redistribution of bodily resources via the actions of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS). The SNS induces the production of short-lived, locally acting neurotransmitters and neuropeptides, while the HPA axis is responsible for the production of various endocrine hormones, including glucocorticoids (GC) and endorphins. These hormones act together to regulate an organism’s physiology and behavior during times of stress, such that increased energy becomes available at the expense of other bodily functions (Reviewed in [1]).

The immunomodulatory effects of psychological stress have been well documented [2]. Although a brief exposure (less than 2 hr) to an acute stressor can lead to enhanced immune responses in the skin [3], prolonged exposure to stress is often immunosuppressive [4]. Chronic stress impairs the efficient generation of CD8⁺ T cell responses, resulting in prolonged courses of infection and increased morbidity and mortality from infections in which CD8⁺ T cells provide protection, such as herpes simplex virus (HSV) and Theiler’s murine encephalitis virus (TMEV) [5, 6]. While several studies have examined the effects of stress on T cells and T cell function [7-9], T cells require dendritic cells (DC) to present peptide antigen in the context of major histocompatibility complexes (MHC) to induce T cell activation [10]. Despite the important role of DC, the effects of stress on DC function and the consequences of such effects have only recently been addressed. The studies described in this dissertation aim
to establish DC as an important target of stress-induced hormones and to elucidate the consequences of impaired DC function on antiviral immune responses and viral pathogenesis.

DC act as a bridge between innate and adaptive immunity. As innate immune cells, DC exist in peripheral tissues, such as cutaneous or mucosal sites, taking up antigen non-specifically. DC become activated by the recognition of a variety of danger-associated molecular patterns (DAMP). Activated DC begin to secrete cytokines, migrate to lymph nodes (LN), and undergo a maturation process enabling DC to efficiently present antigen to T cells [11]. The interaction between mature DC and antigen-specific T cells is critical in the efficient generation of a subsequent T cell response.

It has been established that stress-induced glucocorticoids (GC), including corticosterone (CORT), the glucocorticoid produced in rodents, play an important role in suppressing antiviral cytotoxic T lymphocyte (CTL) responses against HSV, leading to increased mortality and viral replication [12]. However, the effects of CORT on DC, which are required to initiate CTL responses, are relatively unknown. Previous findings have shown that CORT suppresses antigen processing and presentation by a virus-infected DC-derived cell line, suggesting that DC function can be modulated by CORT [13]. Chapter III of this dissertation describes a series of studies that determined how CORT affects the ability of bone marrow-derived DC (BMDC) to respond to stimulation with bacterial lipopolysaccharide (LPS) and prime naive CD8$^+$ T cells. These studies found that CORT impaired the ability of DC to mature in response to LPS stimulation. CORT-treated DC were unable to downregulate endocytosis and were also impaired in
their production of pro-inflammatory cytokines including interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-α. Furthermore, CORT rendered DC less efficient at priming naive CD8⁺ T cells when adoptively transferred in vivo, demonstrating that stress-induced GC are able to suppress DC function.

The demonstration that exogenous CORT can suppress the function of in vitro-generated DC does not mean that stress-induced CORT modulates DC function in vivo. Furthermore, while stress-induced CORT is able to suppress CTL responses against HSV, it is unknown whether this suppression is due to the direct effects of CORT on T cells themselves or due to effects on other cells that are required for proper T cell function, such as DC. The studies described in Chapter IV demonstrate the important role of the effects of CORT on non-T cells in suppressing CD8⁺ T cell responses to HSV infection. Furthermore, these studies also demonstrate that stress is able to suppress DC function in vivo during a viral challenge. The findings presented in this chapter underscore the sensitivity of early events in antiviral immune responses to stress-induced GC and illustrate how stress-induced impairment of these early events can lead to serious consequences at later times during an infection, several days after stress has been terminated, when CORT levels have subsided [14].

Overall, the findings presented in this dissertation demonstrate how DC function is compromised by stress-induced GC. In addition to effects of GC and other neuroendocrine mediators on T cells themselves [15], GC-induced changes in DC function contribute significantly of the effects of stress on the suppression of antiviral T cell responses. Future studies will be required to fully identify other potential cellular targets affected by stress-induced GC and to determine the role of these cells in stress-
induced immunosuppression. While ongoing studies aim to characterize the subcellular mechanisms that underlie the effects of CORT on DC, the findings presented in this dissertation provide evidence that elevated concentrations of CORT have substantial effects on DC function and that the effects of stress-induced CORT act on DC in vivo to suppress the generation of antiviral CTL responses.
CHAPTER II: LITERATURE REVIEW

A. Immune Responses

The function of the immune system is to protect an organism from disease caused by foreign pathogens or the uncontrolled proliferation of malignant tissue. To cope with the diverse array of threats, the mammalian immune system has two complementary strategies to defend against disease. The innate immune system is rapidly mobilized by the recognition of highly conserved DAMP by pattern recognition receptors (PRR). These receptors recognize not only the biological products produced by various foreign microbes, but also endogenous “danger” signals that are produced or released by dying or damaged tissues. The triggering of PRR leads to the production of various chemical mediators to destroy or inactivate pathogens while simultaneously initiating intercellular communication to promote immune cell recruitment, apoptosis of infected cells, and increased resistance to infection by uninfected cells. One result of this intercellular communication is the activation of an adaptive immune response that is specifically targeted to inactivate or destroy the pathogen or pathogen-infected cells. While adaptive immune responses require more time to initiate than innate responses, adaptive immune responses are antigen-specific and thus can be more potent with less of the collateral damage associated with a prolonged non-specific innate immune response.

1. Innate Immunity

a. Pathogen Recognition

Innate immunity consists of the activation and recruitment of various phagocytes, non-specific natural killer (NK) cells, and antigen-presenting cells (APC). These cells
are activated by the triggering of various PRR, expressed on the cell surface, in intracellular vesicles, or in the cytosol. Both parenchymal and immune cells express several PRR [16], allowing for the efficient detection various classes of pathogen. Receptors for bacterial products are expressed at the plasma membrane and detect lipoproteins and lipoteichoic acid (TLR2) [17, 18], LPS (TLR4) [19], and flagellin (TLR5) [20]. Other TLR, expressed in late endosomes and lysosomes, detect the nucleic acids of intracellular or endocytosed pathogens, including dsRNA (TLR3) [21], ssRNA (TLR7) [22], and DNA (TLR9) [23]. Nucleic acids can also be detected in the cytosol by retinoic acid-inducible gene (RIG)-I [24] or melanoma differentiation-associated gene 5 (MDA5) [25], both of which recognize RNA, or the cytosolic DNA receptors DNA-dependent activator of interferon regulatory factor (DAI) [26] and absent in melanoma-2 (AIM2) [27, 28].

The triggering of various PRR leads to the activation of diverse signaling pathways culminating in the production of cytokines, chemokines, and defensive molecules. All of the TLR (with the exception of TLR3) signal through the adaptor protein myeloid differentiation gene 88 (MyD88) [20, 29-32]. TLR-induced MyD88 signaling leads to activation of nuclear factor-κB (NFκB) [33] and the mitogen-activated protein (MAP) kinases, p38 and JUN N-terminal kinase (JNK) [34, 35]. The activation of these signaling pathways is important for the rapid production of pro-inflammatory cytokines in response to pathogen or damage-associated danger [34]. In addition to MyD88, TLR4 signals through Toll/IL-1 receptor-domain-containing adaptor protein inducing IFNβ (TRIF), which is also the sole adaptor protein used by TLR3 [36,
TRIF signaling leads to the activation of NFκB and IFN regulatory factor (IRF)-3 [37], which induces IFNβ synthesis [38].

Cytosolic nucleic acid receptors also induce signaling cascades to initiate immune responses. The RNA receptors, RIG-I and MDA5, like TRIF, lead to the activation of NFκB and IRF3 [24, 25]. The detection of cytosolic DNA by DAI leads to IFNβ production through the activation of IRF3 [26], and AIM2 activation induces the formation of a pro-inflammatory molecular complex known as the inflammasome, resulting in the activation of NFκB and caspase-1 [27, 28]. Caspase-1 activity is required for the secretion of the pro-inflammatory cytokines IL-1 and IL-18 [39, 40]. The myriad of signals induced by diverse microbial or damage-induced stimuli allow the innate immune system to quickly and specifically to a wide range of threats.

b. Effectors of Innate Immunity

The various cytokines and other mediators have critical roles in the initiation and promotion of immune responses. Pro-inflammatory cytokines, such as IL-6, facilitate the activation and proliferation immune cells [41]. Others, such as tumor necrosis factor (TNF)-α, promote immune cell recruitment by activating the expression of adhesion molecules on vascular endothelium [42]. Chemokines induce migration of the appropriate immune cells to sites of infection, such as monocyte chemotactic protein (MCP)-1 [43], or to secondary lymphoid tissues, such as CCL21 [44]. Other immune molecules possess antimicrobial activity, such as β-defensins, which can directly kill certain pathogens [45].
The cells of the innate immune system have diverse functions. Neutrophils and macrophages are able to engulf extracellular pathogens in a process known as phagocytosis. Phagocytosed materials are then degraded in phagolysosomes by proteases [46]. NK cells are able to detect the aberrant expression of cell surface molecules by target cells, most often a lack of MHC class I, and induce target cell apoptosis, thereby eliminating a potentially infected or transformed cell [47]. Innate immunity also encompasses APC, which non-specifically collect, process, and present antigen to T cells in the context of MHC class I and II molecules. DC are the most potent APC and will be discussed in great detail below. In addition to their role in T cell priming, many APC, including DC synthesize cytokines that promote inflammation and rapidly contribute to innate immune responses against pathogens and tumors.

c. Innate Antiviral Immunity

As the studies presented in this dissertation are chiefly concerned with antiviral immunity, it is important to consider how the innate immune system detects and responds to viral infection. Several defensive strategies have evolved to protect the host against viral pathogens. To eliminate a viral infection, steps must be taken to control the production of infectious virus by killing infected cells or shutting down the production of infectious virus, while simultaneously preventing uninfected cells from becoming infected.

The detection of viral pathogens best characterized by the sensing of nucleic acids by intracellular receptors, including Toll-like receptors (TLR) expressed in vesicles and cytosolic receptors, such as DAI. In the case of HSV, viral DNA is detected in
endosomes by TLR9 [48], which is required for innate immunity against HSV [49]. Independently of TLR, HSV is sensed by an unidentified receptor that signals through mitochondrial antiviral signaling protein (MAVS), an adaptor protein utilized by the cytosolic nucleic acid receptors RIG-I, MDA5, and DAI [50]. Besides the detection of HSV nucleic acid, an unidentified surface component of HSV virions triggers recognition through TLR2 on the plasma membrane [51]. The detection of viruses by diverse receptors induces the activation of signaling cascades to initiate the innate immune responses.

One of the most important components of the innate antiviral response is the production of IFN. IFN was first discovered in 1957 as a substance that interferes with the ability of viruses to infect permissive cells [52]. Since the discovery of IFN it has been determined that there are three distinct classes of interferons, each class utilizing its own receptor. Type I IFN include fourteen isoforms of IFNα (13 in humans) and one isoform of IFNβ, IFNε, IFNκ, and IFNω [53]. Although little is known about the induction of IFNε, IFNκ, and IFNω, nearly all cells produce IFNβ in response to viral infection. IFNβ (and other type I IFN) bind to the IFNα/β receptor, and induce the phosphorylation of signal transducer and activator of transcription (STAT)-1 [54]. This leads to the induction of several genes, which promote immune responses and impair viral replication. Specifically, protein kinase R (PKR) [55] and 2′-5′ oligoadenylate synthetase (OAS) are induced which act together to shut down gene expression in virally infected cells [56]. STAT-1 signaling in non-infected cells amplifies innate immunity by inducing the synthesis of IRF-7 [57], which is required for the induction of the various isoforms of IFNα [58], and the upregulating TLR and other PRR, thereby increasing the
sensitivity of cells to “danger” [59]. Type I IFN also promotes innate and adaptive immunity by activating NK cells [60] and promoting T cell priming [61]. IFNγ is the sole type II IFN and is produced by lymphocytes, including CD8+ T cells, Th1 CD4+ T cells, and NK cells. IFNγ promotes inflammation through several mechanisms, notably by activating macrophages [62]. The recently described type III IFN family consists of IFNλ1 and IFNλ2/3 (also known as IL-28 and IL-29) [63, 64]. The production of these proteins has been poorly characterized, although it is known that they can be induced in response to ssRNA and type I IFN. Like all IFN, type III IFN have anti-viral effects in vitro. Although their role in immune responses in vivo is relatively unknown, it has been shown that the administration of recombinant IFNλ can reduce viral loads in HSV-infected mice [65].

The importance of innate immunity to viral infection is underscored by cases of patients with genetic deficiencies in the detection and innate responses to viruses. Patients with homozygous mutations in STAT-1 succumb to lethal viral diseases, including HSV-induced encephalitis [66]. Cells obtained from these patients are completely refractory to stimulation by either type I or type II IFN, demonstrating the crucial importance of IFN-induced signaling in response to viral infections. Notably, STAT-1-deficient patients also suffered from chronic mycobacterial infections, similar to other patients whose cells do not respond to IFNγ [67, 68]. However, the STAT-1-deficient patients alone succumbed to viral diseases, highlighting the critical antiviral role of type I IFN. Patients with genetic defects in the ability to detect viruses also suffer from exacerbated disease. Unc93B is an endoplasmic reticulum (ER)-associated transmembrane protein that is required for proper localization and signaling of endosomal
TLR (3, 7, and 9) [69]. Patients that are homozygous for mutated forms of Unc93B are highly susceptible to HSV-induced encephalitis [70]. Although cells from these patients are unable to synthesize type I and type III IFN in response to viral stimuli, the provision of exogenous type I IFN restored the ability of mutant cells to control virus infection.

Although innate immunity is essential for protection from several pathogens, it is generally insufficient to completely clear an infection. Antigen-specific adaptive immune responses are often required to successfully eliminate invading microbes. The connection between these two arms of the immune system can be found in APC.

2. Antigen-Presenting Cells

APC act at the interface between the innate and adaptive immune responses. In addition to their innate functions of antigen uptake and cytokine production, APC play important roles in the initiation of adaptive immune responses. Broadly, APC include any cell that is capable of presenting antigen to T cells; however the term APC most often refers to professional APC (pAPC), which are a class of cells that constitutively express MHC class II and are specialized to present antigen to naive T cells. Under the appropriate conditions, the interaction between antigen-bearing pAPC and naive T cells leads to T cell activation. pAPC include DC and activated macrophages and B lymphocytes. Of these cells, DC are highly efficient at acquiring, processing, and presenting antigen to T cells and are the most potent inducers of T cell activation [71, 72]. Conventional T cells, expressing the αβ-T cell receptor (TCR), recognize peptide antigen presented in the context of MHC class I or II. CD8+ T cells recognize 8-10 amino acid peptides presented in the context of MHC class I, while CD4+ T cells
recognize longer peptides, usually 13-17 amino acids long, presented in the context of MHC class II [73]. Naive T cells require additional signals for proper activation, otherwise they will become “anergic” or unresponsive to subsequent antigen presentation. These signals are generally delivered in the form of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) expressed on the surface of an activated APC. B7.1 and B7.2 can ligate the CD28 receptor on T cells, providing the “second signal” required for T cell activation.

a. Introduction to DC

The first description of what are now known as DC was published in 1868, when Paul Langerhans observed cells with long dendritic processes in the epidermal layer of human skin, which he suspected were nerves for the skin [74]. Over 100 years later, Steinman and Cohn identified a small population of large cells with a dendritic morphology found in the white pulp of the mouse spleen [75]. Since the “rediscovery” of DC in 1973, knowledge about these cells has exploded exponentially.

Although, initially found in LN, Peyer’s patches and the white pulp of the spleen [75], DC have been identified in several parenchymal tissues including the skin, liver, thyroid, and connective tissues throughout the body [76]. Further experimentation by Steinman led to the discovery that DC possess potent immunostimulatory properties and are far more efficient than other splenic cells, such as macrophages and lymphocytes, in the stimulation of mixed-lymphocyte reactions [77]. Soon after these discoveries, DC were identified as “accessory cells” that are critical for stimulating CTL [71] and T\textsubscript{H} cell responses [72]. Interestingly, the role of DC in T cell “stimulation” was initially unclear,
but it was thought that they were NOT antigen presenting cells as they exhibited little ability to take up exogenous antigen after \textit{ex vivo} isolation [71]. Their antigen presenting capacity was eventually confirmed when it was found that purified DC of the proper MHC haplotype could be used as the sole stimulators to induce IL-2 production by an antigen-specific T cell hybridoma [78].

It is now known that DC can be classified into a myriad of subsets with diverse phenotypic and functional characteristics. Conventional DC (cDC) consist of lymphoid-resident DC, DC that are found in lymphoid tissues in the steady-state, and migratory DC, which exist in peripheral tissues and migrate to LN in the steady-state or during inflammation [79]. cDC are distinct from plasmacytoid DC (pDC), a subset of DC that can quickly produce large amounts of type I IFN [80], and the so-called IFN-producing killer DC (IKDC), a recently described subset of cells which possess properties of both DC and NK cells [81].

The life history of an individual DC varies greatly from one subset to the next. Most DC, however, do have several properties in common. In the mouse, DC are typically identified by their expression of the integrin CD11c [82] and MHC class II. However, CD11c can also be expressed by lung macrophages [83], activated T cells [84], certain plasma cells [85], and a recently described set of NK cells [86-88].

b. DC Ontogeny

The relatively small number of DC found in any given tissue (comprising 1-2% of the cells of the spleen in the steady state [75]) and the diverse array of DC subsets have made it difficult to elucidate the various pathways of DC differentiation. Like other cells
of the immune system, DC originate from bone marrow-derived hematopoetic stem cells (HSC) [89]. Unlike other immune cells, DC can follow several pathways from stem cell through terminal differentiation. Whereas neutrophils and macrophages differentiate from common myeloid progenitors (CMP) [90], and B and T lymphocytes develop from common lymphoid progenitors (CLP) [91], DC are able to differentiate from both CMP and CLP [92, 93].

While the exact steps of differentiation from CLP to DC remains unclear, recent work has identified a series of precursor cells in progressive stages along the pathway of differentiation from CMP to DC [94]. Cells from each successive stage of this pathway are increasingly limited in their ability to differentiate into multiple cell types. It has long been understood the CMP can give rise to DC [95] in addition to monocytes, granulocytes, megakaryocytes, and erythrocytes [90]. These cells do not express lineage (Lin) markers (CD3, CD19, Ter119, NK-1.1, and B220), or stem cell antigen (Sca)-1, but express high levels of c-Kit, the receptor for the cytokine stem cell factor [90]. Some CMP express high levels of the fractalkine receptor (CX3CR1) and can differentiate into DC and cells of the monocyte/macrophage lineage, but not granulocytes of other myeloid cells [96]. These DC/monocyte progenitors differentiate into fms-like tyrosine kinase 3 (FLT3)$^+$ macrophage-colony-stimulating factor receptor (M-CSFR, CD115)$^+$ c-Kit$^{lo}$ cells that are able to differentiate into cDC and pDC but do not give rise to monocytes [97, 98]. These DC-restricted progenitors exist in the bone marrow where they become pDC and CD11c$^+$ MHC class II$^+$ cDC-restricted precursors. These cDC-precursors are unable to become pDC but migrate through the bloodstream to lymphoid organs where they can quickly differentiate into conventional lymphoid-resident DC [94].
Two cytokines have been implicated in DC differentiation, FLT3 ligand (FLT3L) and granulocyte-macrophage-colony-stimulating factor (GM-CSF). FLT3L is required for differentiation of all types of DC in the steady-state [99]. FLT3L binds to FLT3 and signals through STAT3 to induce DC differentiation [100]. Mice that receive FLT3L, either exogenously or from implanted FLT3L-secreting tumors [101], possess extremely high numbers of all types of DC in secondary lymphoid tissues [102]. GM-CSF is also known to induce DC differentiation and has long been used in the in vitro generation of DC from bone marrow [103]. However, it has recently been appreciated that GM-CSF is likely to be more important in promoting DC differentiation under inflammatory conditions than in the steady-state [104], suggesting that DC generated in vitro with GM-CSF may more closely resemble monocyte-derived DC than DC that differentiate in the steady-state [105].

c. Properties of DC

i. Immature DC

After differentiation, most DC (in lymphoid organs or peripheral tissues) exist in an immature state, in which they are highly efficient at taking up antigen but relatively poor at priming naive T lymphocytes [106]. Immature DC sample their environment via mannose receptor-mediated endocytosis and macropinocytosis or fluid-phase uptake [107]. Although immature DC synthesize MHC class II, this protein is retained intracellularly in lysosomes [108] due to its association with MHC class II invariant chain (Ii) [109]. Immature DC do not express high levels of the classical costimulatory
molecules, B7.1 (CD80) and B7.2 (CD86) [110]. Consequently, interactions between immature DC and naive T cells lead to an anergic state [111].

In the steady-state, a small number of immature DC migrate to secondary lymphoid tissues from the periphery [112]. These DC, as well as immature lymphoid-resident DC, can present self-antigens to auto-reactive T cells and are thought to promote peripheral tolerance to host tissues [113].

**ii. DC Maturation**

Immature DC respond to diverse infectious or inflammatory stimuli by undergoing a series of functional and phenotypic changes known as maturation. These stimuli may be microbial in nature, such as bacterial LPS [114] or viral DNA [48], or they may be products released by damaged or dying cells, such as high mobility group box protein-1 [115]. In addition to direct activation by DAMP, DC can also be activated other activated immune cells. Several types of immune cells produce TNFα during inflammation, which acts on DC to induce DC maturation [116]. Activated CD4+ T cells express CD40L, which can also stimulate DC to become immunostimulatory [117].

DC maturation is characterized by several functional and phenotypic changes that enable DC to potently activate or regulate T cell responses. In response to inflammatory or infectious stimuli, maturating DC will transiently increase their rate of antigen uptake for several hours before reducing uptake dramatically, allowing DC to “load up” with pathogen-associated antigen acquired near a site of infection [118]. Maturing DC upregulate the chemokine receptor CCR7, enabling them to migrate to the T cell areas of draining LN [119]. The steady-state trickle of immature DC migrating to draining LN
increases several-fold in the presence of an inflammatory stimulus [120]. The hallmark of DC maturation is the upregulation of costimulatory molecules, B7.1 and B7.2 [117], and the downregulation of the cathepsin inhibitor cystatin C, which permits the trafficking of MHC class II molecules to the plasma membrane [109]. In addition, these DC produce several pro-inflammatory cytokines that promote innate and adaptive immunity [121]. Together, these changes enable mature DC to take up, process, and present pathogen-associated antigen to T cells in LN, resulting in efficient T cell activation.

DC can also undergo a non-inflammatory maturation process in response to changes in signaling from intercellular adhesion molecules. The disruption of homotypic interactions between E-cadherin expressed on DC and neighboring cells induces an alternative maturation program in DC that is mediated by β-catenin signaling [122]. DC that undergo this alternative maturation display several changes that are similar to those that occur during “classical” DC maturation. These DC also downregulate macropinocytosis and have increased surface expression of MHC class II, costimulatory molecules, and CCR7, but unlike DC that mature in response to infection, alternatively-matured DC do not produce-inflammatory cytokines. In addition, although these DC are able to present antigen to T cells and induce T cell proliferation, T cells primed by alternatively-matured DC do not possess effector functions but rather resemble regulatory T cells (Treg). The disruption of homotypic E-cadherin interactions in cultures of bone marrow-derived DC (BMDC) has been proposed as the cause of “spontaneous” maturation that has been observed in vitro [122]. In vivo this pathway of maturation may
be a mechanism by which DC actively promote tolerance to self-antigens to prevent autoimmunity [122].

iii. Antigen Presentation

Interactions between DC and antigen-specific T cells depend on the processing and presentation of antigen. Antigen is presented to CD8$^+$ T cells via two distinct pathways. Direct presentation occurs when protein antigen is synthesized within an APC. In the direct presentation pathway, antigen is enzymatically degraded by the proteosome [123] and small peptides are transported into the ER by the transporter associated with antigen processing [124, 125]. In the ER, peptides are further trimmed by additional peptidases [126] and loaded onto MHC class I molecules to be transported to the plasma membrane [127]. However, successful antigen presentation by the direct pathway requires APC to be infected. Furthermore, this pathway will not work when the infecting pathogen actively suppresses antigen presentation, as occurs during certain herpesvirus infections [128]. To circumvent these potential problems, certain subsets of DC utilize an additional antigen presentation pathway, known as cross-presentation. Cross-presentation occurs when DC take up exogenous antigen, which is released into the cytosol, becoming available to the direct presentation pathway [129]. The molecular forms of cross-presented antigen remain unclear but are thought to include dead or dying cells or large portions of relatively intact proteins [130, 131].

Traditionally it was understood that the primary source of antigen for MHC class II is exogenous protein that had been taken up and degraded in lysosomal compartments. Cathepsins degrade these proteins into peptides to be loaded onto MHC class II
molecules and transported to the plasma membrane [132]. It is now known that endogenously produced proteins are made available for presentation on MHC class II via autophagy, a process by which cells sequester a portion of their cytosol for lysosomal degradation [133]. Both of these pathways are utilized by DC in the generation of CD4+ T cell responses against foreign antigens and the promotion of tolerance to self-antigen.

d. DC Subsets

i. Conventional DC

cDC can be classified into two broad categories: lymphoid-resident DC and migratory tissue DC [104]. Resident DC are derived from blood-borne precursors that differentiate into DC in the spleen and LN [134]. Migratory DC differentiate in peripheral tissues and constitutively migrate to draining LN in the steady-state [135]. In addition to their differing anatomical locations, these two DC subsets have distinct but complementary roles in initiating and regulating adaptive immune responses.

i-a. Lymphoid-resident DC

Lymphoid-resident DC can be further classified by their expression of CD4 and the α chain of CD8. CD8α+ CD4- DC are found in the T cell zone of the spleen and LN and are the predominant DC of the thymus [136]. The CD8α+ subset is the only subset of DC that can efficiently cross-present exogenous antigen to CD8+ T cells in vivo [137, 138] and is also characterized by the ability to produce large quantities of IL-12 [139]. Several studies have found that CD8α+ DC are critical for priming CD8+ T cells during viral infection [140, 141] and can prime CD4+ T cell responses as well [142]. The requirement for CD8α+ DC is elegantly demonstrated in mice that are deficient for the
transcription factor Batf3. These mice possess all known subsets of DC with the exception of the CD8α+ subset [143]. Batf3−/− mice are strongly impaired for their ability to mount CD8+ T cell responses and are unable to cross-present antigen from viruses or tumors.

The function of lymphoid-resident CD4+CD8− or double-negative (DN) DC remains somewhat unclear. These cells express the CD11b integrin and may have a role in presenting antigen to CD4+ T cells in the context of MHC class II [144], but this may depend on the route of infection and the accessibility of antigen to the draining node [145]. Little is known of the function of CD4+ DC, which are found primarily in the spleen [136], although they may contribute to the priming of CD4+ T cells [142].

**i-b. Migratory DC**

Migratory DC represent the classical interstitial DC. This subset of DC is located in peripheral tissues where they exist in an immature state and act as sentinels. Upon their encounter with a pathogenic or inflammatory stimulus, migratory DC begin to mature and migrate to secondary lymphoid tissues through afferent lymphatics where they present antigen acquired in the periphery to T cells. An example of these cells includes the lung-resident DC, which are highly efficient at stimulating both CD4+ and CD8+ T cells after migrating to mediastinal LN in a model of influenza infection [145]. The efficiency of antigen presentation by migratory DC may depend on the pathogen and route of infection examined. In a cutaneous model of HSV infection, migratory DC were unable to present antigen to CD8+ T cells, but were required to carry antigen from the site of infection to cross-presenting DC in the LN [146]. Migratory “dermal DC” have been subsequently reported as also able to present antigen to CD8+ T cells during cutaneous
infections, albeit with much less efficiency than CD8α⁺ DC [142]. Langerhans cells (LC) of the epidermis were eventually confirmed to be another example of migratory DC [147, 148]. The role of LC in T cell priming is controversial; however, they too may be important for transporting antigen to LN [149].

ii. Plasmacytoid DC

Plasmacytoid DC (pDC) are a unique subset of rare cells that are capable of rapidly producing large quantities of type I IFN without prior stimulation [80]. The discovery, identification, and characterization of pDC spanned several decades. pDC were first described as cells in the T cell areas of human LN with the morphology of antibody-secreting plasma cells [150]. These cells do not express surface immunoglobulin but are CD4⁺ and were initially called plasmacytoid T cells [151]. Further examination revealed that “plasmacytoid T cells” were CD3⁻ but MHC class II⁺, thus casting doubt on their T cell lineage [152]. The identity of the plasmacytoid cells remained a mystery until it was found that treatment with IL-3 and CD40L induced their differentiation into CD11c⁺ cells with a dendritic morphology that could strongly induce allogeneic T cell proliferation [153]. Eventually, it was recognized that pDC resembled descriptions of previously described, but unidentified non-T cells capable of producing large amounts of type I IFN [154]. pDC were confirmed to be the same cells previously described as natural IFN-producing cells when it was observed that a preparation of CD4⁺, lineage⁻, CD11c⁻ cells sorted from human blood was capable of producing > 200-fold more type I IFN in response to HSV-stimulation than several other cell types [80].
An analogous population of pDC has subsequently been identified in the mouse as CD11c<sup>lo</sup>, B220<sup>+</sup>, Ly6C<sup>+</sup> cells [155-157].

pDC are likely to have evolved for the detection of viral pathogens as they express endosomal TLR7 and TLR9, which detect ssRNA and DNA, respectively (no 78; Lund et al., 2003 JEM) [22, 158]. In contrast to other immune cells, pDC are able to rapidly produce various forms of IFN, including all forms of IFNα, IFNβ, IFNω, and the type III IFN, IFNλ [159]. IFN mRNA can be detected in pDC within 4 hr after stimulation, reaching its peak after 12 hr; most type I IFN is produced within the first 24 hr following stimulation, with pDC producing 3-10 pg/cell [80]. It is thought that the constitutive expression of IRF-7 allows pDC to rapidly produce the various type I IFN without the need for a productive viral infection [160].

After being triggered to produce IFNα, pDC begin to mature [161]. Maturing pDC gain the ability to process and present antigen in vitro, but their role in antigen presentation in vivo is controversial [162]. Recent studies have found that, unlike cDC, pDC are able to continue to present endogenous synthesized antigen to CD4<sup>+</sup> T cells via MHC class II, well after maturation occurs [163]. In addition pDC are able to prime CD4<sup>+</sup> T cells in LN in vivo [164]. However, pDC are unable to prime CD8<sup>+</sup> T cells in vivo [164], and presentation by pDC to CD4<sup>+</sup> T cells is not required for protection in models of mucosal HSV and lung influenza virus infections [49, 145], suggesting that their principle role in immunity is through the production of type I IFN.
iii. IFN-producing Killer Cells

A controversial subset of DC was recently described to possess markers and properties of both NK cells and DC. CD11c\textsuperscript{lo}, B220\textsuperscript{−}, MHC class II\textsuperscript{+} cells were identified in mouse spleen [81]. These cells express the NK cell markers CD49b, NKG2D, and NK1.1 (in NK1.1\textsuperscript{+} strains). Although their morphology resembles neither DC nor NK cells, these cells were initially classified as DC, based on their expression of MHC class II, B7.1, and B7.2, which are not expressed by classical NK cells. After stimulation with CpG-DNA, these cells gain potent cytotoxic properties, killing target cells in an NKG2D-dependent manner, and produce large amounts of IFN\textgreek{a} and IL-12. By 45 hr post-stimulation, however, these cells lose their cytotoxic properties and upregulate MHC class II, B7.1, and B7.2 and gain the ability to weakly present antigen to T cells [81]. Subsequent studies on these cells by several independent investigators have refuted their classification as DC by demonstrating their poor antigen presenting abilities, failure to produce of IFN\textgreek{a} after stimulation, and dependence on IL-15, a cytokine that promotes NK cell differentiation [86-88]. The so-called “IFN-producing killer DC” more likely represent a subset of B220\textsuperscript{+}, CD11c\textsuperscript{lo} NK cells. The controversy surrounding the discovery and classification of these cells illustrates the high degree of complexity regarding the identification and characterization of the many DC subsets.

e. Role of DC in Antiviral Immunity

The diverse subsets of DC have distinct but complementary roles on protecting the host from viral infections by contributing to innate and adaptive immune responses. pDC are particularly important for innate immunity, due to their ability to quickly
produce large amounts of type I IFN in response to intracellular viral nucleic acid [48]. In an intravaginal model of lethal HSV-2 infection, pDC were recruited to the vagina over the first 4 days following infection, possibly via the chemokine receptors CCR5 and CXCR3, and produced large amounts of IFNα [49]. When pDC were depleted, infected mice suffered from an accelerated rate of mortality [49].

While pDC appear unable to prime CD8+ T cell responses [141, 165], they may have other roles in T cell activation. In a cutaneous model of HSV infection, pDC can stimulate cDC in a contact-dependent manner and through the provision of IFNα [61]. Together these pDC functions can thought to promote T cell priming by cDC, although this may depend on the routes of infection in which pDC are allowed to come in contact with draining antigen. In a mucosal model of HSV infection, depleting pDC had no effect on CD8+ T cell activation [49].

Given the diversity of cDC subsets, the technical difficulties in discriminating between lymphoid-resident and migratory DC, and the diverse infectious model employed by different investigators, it may not come as a surprise that a consensus has not been achieved as to which subsets of cDC prime T cell responses in vivo. However, it is clear that LN-resident CD8α+ DC are capable of priming CD8+ T cells in response to cutaneous and mucosal viral infections [141, 165]. The importance of this subset of cross-priming DC is clearly demonstrated by the aforementioned Batf3-deficient mice, which lack CD8+ DC and have severely impaired CD8+ T cell responses to viral infection [143]. However, the role of CD8- DC in CD8+ T cell priming is highly controversial. Originally it was found that CD8- DC were unable to present viral antigen to CD8+ T cells in a model of cutaneous HSV infection [140]. Subsequently, it was discovered that
a subset of migratory CD8\(^+\) DC are able to present viral antigens from infections originating in mucosal tissues, including the airway [166] and vaginal epithelium [165]. Furthermore, although migratory CD8\(^-\) DC are reportedly unable to present antigen to CD8\(^+\) T cells in from a cutaneous route of infection, they are thought to be required to “carry” antigen from the site of infection to draining LN where it is transferred to resident CD8\(\alpha^+\) DC for CD8\(^+\) T cell priming [146]. Adding to this complexity is the recent finding that a LN-resident CD8\(^-\) DC population also primes CD8\(^+\) T cells in a cutaneous model of HSV infection [165]. The discrepancy between this finding and the previous finding that CD8\(^-\) DC are not able to prime CD8\(^+\) T cells in cutaneous infections was explained by the presence of contaminating CD11c\(^+\) NK cells that suppressed T cell priming when co-purified with CD8\(^-\) DC in certain DC subset enrichment strategies.

The DC subsets that are responsible for priming CD4\(^+\) T cell responses to viral infections have also been described, although these findings like those for cells that prime CD8\(^+\) T cells likely depend on specific routes of infection. For mucosal infections with HSV [165, 167] and influenza virus [145], it is thought that migratory CD8\(^-\) DC are primarily responsible for presenting antigen to CD4\(^+\) T cells. In the airway these DC have been described as langerin\(^+\) CD11b\(^-\) [145], but are thought to be CD11b\(^+\) submucosal DC in the vaginal tract [167]. During cutaneous infections several subsets have been implicated in priming CD4\(^+\) T cell responses. LN-resident DC (CD8\(^+\) and CD8\(^-\)) appear to be responsible for the bulk of antigen presentation to CD4\(^+\) T cells during cutaneous infections with HSV [165]. Whereas, LN-resident CD8\(^+\) DC (but not CD8\(^-\) resident DC) and migratory dermal DC were found to be responsible for CD4\(^+\) T cell priming in a cutaneous model of influenza virus infection [142]. Antigen
presentation to CD4$^+$ T cells during systemic infection is mediated by all subsets of spleen-resident DC, with an important role for CD4$^+$ DC [142]. Although pDC have been found able to prime CD4$^+$ T cell responses in some experimental systems [164], several studies using viral infections have failed to implicate pDC in T cell priming [142, 145, 165].

In addition to their role in T cell priming it is likely that cDC provide essential innate immunity during viral infections. The role of cDC during HSV infection can be determined using CD11c-DTR mice, whose cDC (CD11c$^{hi}$) can be depleted by the exogenous administration of diphtheria toxin [168]. cDC depletion results in 100% mortality from a cutaneous infection with a non-lethal dose of HSV-1 within 6 days [168]. An examination of viral spread found that cDC-depleted mice have increased viral infiltration of the spinal cord by day 4 post-infection, suggesting impaired innate immunity. This innate immunity provided by cDC is distinct from innate immunity provided by pDC as pDC are not depleted from CD11c-DTR mice [164].

3. Adaptive Immunity

Adaptive immune responses consist of the mobilization and differentiation antigen-specific T and B lymphocytes into effector cells. Although adaptive immune responses require more time to initiate than innate immune responses, they are highly antigen-specific, allowing for a more vigorous response than could be sustained by an innate immune response with less collateral damage. Adaptive immune responses also lead to immunological memory. The activation of naive lymphocytes induces a subset of these cells to expand and differentiate into memory cells that will be ready to respond
quickly to subsequent infections by the same. This larger memory population enables for a more rapid and vigorous response to subsequent infections.

The cells of the adaptive immune system include T and B cells. Most T cells in mouse and human adults express the conventional \( \alpha \beta \)-TCR and a co-receptor for MHC molecules, either CD4 or CD8. CD4\(^+\) T cells recognize antigen presented in the context of MHC class II and usually differentiate into helper T (T\(_H\)) cells upon activation. T\(_H\) produce cytokines that can promote, modulate, or suppress the ongoing immune response. T\(_H\) cells also express the cell surface protein CD40L, which promotes B cell differentiation and activates macrophages and DC [169]. Some CD4\(^+\) T cells express the transcription factor FoxP3; these cells, known as regulatory T cells (T\(_{Reg}\)) produce immunosuppressive cytokines to control inflammation and dampen the immune response.

CD8\(^+\) T cells recognize antigen presented by MHC class I and differentiate into CTL, which have a role in killing infected or transformed cells. CTL possess perforin and granzymes in intracellular vesicles and the surface protein FASL. Perforin and granzymes can be released directly at a target cell expressing the appropriate peptide-MHC class I to induce apoptosis by caspase activation. FASL binds to the FAS protein expressed by numerous cell types to induce apoptosis through the extrinsic pathway [170].

B lymphocytes possess antibodies as their antigen receptors. Antibodies recognize portions of full-length proteins in specific conformations. Conventional B cells are activated by the recognition of antigen while simultaneously receiving signals from T\(_H\) cells, often via CD40L binding to CD40 on the B cell plasma membrane [169]. Upon activation, B cells proliferate and differentiate into antibody-secreting plasma cells [171].
Signals from cytokines and CD40L cooperate to induce antibody class switching, resulting in the production of antibodies with diverse effector functions, including the binding and neutralization of pathogens, opsonization of extracellular pathogens to promote phagocytosis, and activation of complement proteins to kill pathogens and promote inflammation [172].

**a. Adaptive Antiviral Immunity**

Neutralizing antibodies can prevent infection altogether by preventing viruses from interacting with host cell receptors. In addition to neutralization, virus-bound antibodies can fix complement, thereby inactivating viral particles. To date, every successful anti-viral vaccine relies on eliciting neutralizing antibodies. Although the importance of B cells and antibodies in antiviral immunity has been established a comprehensive discussion of this topic is beyond the scope of this review.

The importance of cytotoxic CD8$^+$ T cells in controlling viral infections has long been recognized [173-175]. When activated by the recognition of a specific peptide-MHC complex on the surface of a mature DC, naive CD8$^+$ T cells begin to proliferate and acquire cytotoxic effector functions. Activated CD8$^+$ T cells respond to chemokines produced during inflammation, such as RANTES (regulated upon activation, normal T cell expressed and secreted) and MCP-1, by migrating to sites of infection [176]. Upon arriving at infected tissues, effector CD8$^+$ T cells recognize viral antigen presented on MHC class I by virus-infected cells. This recognition induces the formation of an immunological synapse, which targets the release of cytotoxic granules, containing perforin and granzymes, directly to the infected target cell [177]. These molecules are
taken up into endosomes by target cells where perforin molecules create small pores in endosomal membranes, releasing granzymes into the cytosol [178]. Granzymes, particularly granzyme B, are able to initiate apoptosis by cleaving pro-apoptotic proproteins [179].

The ability of CD8\(^+\) T cells to provide protection from HSV has long been recognized [174]. Although mice whose CD8\(^+\) T cells are depleted do not die from cutaneous HSV infection, these mice do have higher viral loads and delayed viral clearance [180]. Furthermore, memory CD8\(^+\) T cells have an important role in controlling HSV reactivation from latently infected sensory ganglia [181]. HSV reactivation is prevented by the secretion of granzymes by CD8\(^+\) T cells, which do not lyse infected neurons but can inactivate viral proteins [182].

CD4\(^+\) T cells also play an important role in antiviral immunity. Although inflammatory signals can often stimulate DC such that they are able to prime CD8\(^+\) T cells, additional signals from CD4\(^+\) T\(_H\) cells are required for DC to fully activate CD8\(^+\) T cell responses to certain viruses, such as adenovirus [183]. It is thought that CD40L expressed by activated CD4\(^+\) T cells binds to CD40 on DC to condition DC to efficiently prime CD8\(^+\) T cells [184-186]. CD4\(^+\) T\(_H\) cells are important for efficient immune responses in a dermal model of HSV infection. In the absence of help from CD4\(^+\) T cells, HSV-infected mice have higher viral loads and delayed viral clearance [180]. Although CD8\(^+\) T cells in HSV-infected mice gain cytolytic function without help from CD4\(^+\) T cells, these CTL are impaired for the production of IFN\(\gamma\) and TNF\(\alpha\) [187].

Overall successful control of viral infections depends on the coordination of complementary components of the innate and adaptive immune systems. Innate
immunity is often insufficient to control an infection without an adaptive immune response, but these adaptive responses are dependent on innate immune cells for their induction and regulation. With DC at the center of this interaction of systems it is essential to understand how DC function can be regulated and modulated by the various physiological systems of the body.
B. Neuroendocrine-Immune Interactions

There is a long trail of anecdotal evidence suggesting that interactions occur among the nervous, endocrine, and immune systems. The notion that mood or mental stress can promote or reduce the susceptibility of an individual to infection is widespread. During the past several decades, many studies have begun to elucidate how the nervous system can modulate immunity, which often occurs through the action of various endocrine hormones, neuropeptides, and neurotransmitters [188-191]. Other studies have also investigated how products of immune responses affect the endocrine and nervous systems, resulting in changes in mood, personality, or behavior [192]. Insights into this field, known as neuroimmunology, neuroendocrine-immunology, or psychoneuroimmunology, can lead to an improved understanding of how mental and behavioral situations can effect the appropriate generation of immune responses to immunization, pathogen challenge, self-antigen (in the case of autoimmunity), or innocuous environmental allergens.

Several studies have demonstrated that people who experience chronic psychological stress, such as war-zone refugees [193] or caregivers of dementia patients [194], experience disregulation of the innate and adaptive immune systems. Chronic stress and/or stress-induced hormones have been linked to impaired immune responses against several human pathogens [195, 196] and are associated with a failure of the immune system to control the proliferation of malignant cells in animal models [197]. Paradoxically, while effective immune responses against pathogens are suppressed, chronic inflammatory diseases, such as cardiovascular disease, and allergic diseases, such as atopic dermatitis, are often exacerbated by stress [198].
While it is generally agreed that most forms of chronic stress are immunosuppressive, some studies have shown augmentation of immune responses following brief exposure to acute stress [199, 200]. Brief exposure to stress (less than 2 hr) induces T cells, B cells, NK cells, and monocytes migrate from the blood to cutaneous tissues. This migration is associated with the enhanced generation of skin delayed-type hypersensitivity reactions and may have evolved to prevent infections from developing in wounds sustained during a stressful encounter [201, 202].

1. The Stress Response

When an animal experiences a change in environment or circumstance that is perceived as a threat, a series of physiological changes occurs, known as the stress response [1]. These changes are thought to be directed at reducing threats and returning the body to homeostasis [1]. The stress response consists of activation of the HPA-axis and the SNS [203]. Activation of the SNS results in the local release of catecholamines by neurons, including epinephrine and norepinephrine [204]. The studies presented in this dissertation focus on products of the HPA axis; although SNS-derived hormones have potent immunomodulatory effects [205, 206], they will not be discussed further here.

Activation of the HPA axis occurs when an individual perceives a stressor, inducing the hypothalamus to secrete corticotropin-releasing factor, which acts on the anterior pituitary gland to induce the synthesis of proopiомelanocortin (POMC) [207]. POMC is a polyprotein, which is cleaved into β-endorphin, melanocyte stimulating hormones (MSH), and adrenocorticotropin-releasing hormone (ACTH) [208]. ACTH
enters into systemic circulation and activates the synthesis and release of GC by adrenal glands. Serum concentrations of natural GC (cortisol in humans, CORT in rodents, Fig. 1) are regulated by corticosteroid-binding globulin (CBG), which can sequester endogenously produced GC, reducing their biological availability [209]. In addition, local tissue concentrations of GC can be modulated by the expression of 11β-hydroxysteroid dehydrogenases [210], which catalyze the interconversion of GC between the active (cortisol and CORT) and inactive (cortisone and 11-dehydrocorticosterone) forms [211].

GC are lipophilic steroid hormones that diffuse across the plasma membrane and bind to cytosolic GC receptors (GR) [212]. GR mRNA is expressed in several tissues throughout the body including: thyroid gland, salivary gland, liver, prostate, skin, adipose tissue, heart muscle, CD4+ T cells, CD8+ T cells, B cells, monocytes, pDC, and brain tissue [213]. The binding of GC to GR induces a conformational change in GR enabling GR dimerization [214]. Dimers of GC-bound GR are transported to the nucleus where GR is able to either activate or repress transcription by directly binding to DNA-encoded GC response elements (GRE) [215] or by interacting with other transcription factors [216].

Although the mechanisms by which GC modulate immunity are complex, an understanding of the intracellular targets for GR is beginning to form. In addition to directly activating or repressing transcription via direct engagement of GRE [215], GC-bound GR dimers can interact with other transcription factors, such as AP-1 [216] and NFκB [217]. By binding to these factors, GR can reduce their ability to bind DNA, thus weakening the ability of transcription factors to activate transcription [217, 218]. GC can
**Figure 1.** Molecular structures of (A) cortisol, the GC produced in humans, (B) CORT, the GC produced in rodents, and (C) RU-486, the synthetic GR antagonist.
Figure 1

Cortisol

Corticosterone
induce transcription of the inhibitor of NFκB (IκB), which binds NFκB and retains it in
the cytosol [219, 220]. GR may also modulate NFκB signaling by competing for
cofactors utilized during NFκB transactivation, including CREB-binding protein and
steroid receptor coactivator-1 [221].

The immunosuppressive properties of GC have resulted in their widespread
pharmacological use in attempts to regulate immune responses [222, 223]. Topical
preparations of cortisol are commonly used to relieve cutaneous inflammation [224] and
numerous preparations of oral or inhaled synthetic steroids are often used to treat allergic
asthma [222]. In addition to these uses, GC have also been used as therapeutic
immunosuppressants in preventing transplant rejection and autoimmunity [225, 226].
However, many clinicians are well aware that these uses of GC can often lead to
increased risk of infection, due to immunosuppression.

2. Underlying mechanisms of Stress/GC-induced immunosuppression

As studies in humans have demonstrated the relevance of stress-induced immuno-
modulation, many experiments have been performed in animal models to determine the
mechanisms of this modulation. These studies employed various techniques to model the
perception of psychological stress. Some of these models include physical components
that may directly affect an animal’s physiology, such as footshock [227], cold [228], or
exercise [229]; other models are primarily psychological, including restraint [7], light
[230], and noise-based stressors [231]. The use of these models has promoted an
understanding of the mechanisms underlying stress-induced immunosuppression and
identified GC as a key mediator behind many effects of chronic stress on innate and adaptive immunity to infection [232].

One of the oldest and most widely used methods to model psychological stress in animals is restraint [233]. During restraint stress an animal’s mobility is limited by confinement in a small chamber for a period of minutes to several hours [234]. More severe versions of this stressor employ means, such as securing an animal to a board, to immobilize an animal [235]. Compared to other stressors such as cold-exposure or exercise restraint stress is a more potent inducer of GC responses [236]. In non-stressed mice, serum CORT concentrations vary according to a circadian rhythm, ranging from about 50 ng/ml to 200 ng/ml [237]. Mice that have been subjected to daily 16 hr sessions of restraint stress exhibit significantly elevated concentrations of serum CORT of approximately 500-1000 ng/ml [238]. This elevated CORT concentration declines within 4 hrs of the cessation of the stressor [239]. The restraint method is often used to model stress due to its reliability in inducing a reproducible activation of the HPA-axis, and because it is largely psychological in nature [234]. However, it must be appreciated that the decreased mobility and physical activity may contribute to physiological changes observed in stressed mice.

a. General mechanisms of GC-induced immunosuppression.

Stress and stress-induced GC have pleiotropic effects on many components of the immune system. GR expression has been found in several different types of immune cell, including T and B lymphocytes, monocytes, DC, and neutrophils [213, 240]. Stress-induced GC are known to modulate immunity by at least three distinct mechanisms:
effects on cell viability, recruitment, and function. The effects of a GC on a specific cell are likely to be determined by several factors, including the identity of the cell, the presence or various growth factors, cytokines, or other hormones, the concentration and form (natural vs. synthetic) of the GC, and the duration of GC exposure.

The most direct mechanism by which GC modulate immune function is to induce apoptosis of immune cells. Several types of immune cells are susceptible to GC-induced apoptosis, including monocytes, neutrophils, B cells, and T cells [241]. The apoptosis of DC is induced by the synthetic GC dexamethasone (DEX) [242], but studies using naturally-produced GC have not found any increased apoptosis in CORT-treated DC (see Chapter III). Diverse molecular mechanisms have been implicated in the induction of apoptosis by GC. GC can interfere with certain transcription factors that may be required for cell survival, such as AP-1 signaling [243] and can downregulate expression of the survival factor c-Myc [244]. Developing thymocytes are exceptionally sensitive to GC-induced apoptosis, which is associated with activation of the proteasome, resulting in cleavage and activation of pro-apoptotic mediators [245].

In addition to killing immune cells by apoptosis, GC can prevent the recruitment and migration of immune cells by impairing chemokine synthesis and the expression and activation of adhesion molecules. GC can interfere with the recruitment of phagocytes and other innate immune by impairing the ability of inflamed tissue to synthesize chemokines, such as RANTES and MCP-1. The mRNA encoding MCP-1 is destabilized by GC treatment, downregulating its expression [246], while the synthesis of other chemokines can be impaired by GC-induced suppression of NFκB activity [247].
Adhesion molecules expressed by both leukocytes and parenchymal tissues can be downregulated by GC [248-250].

Even when immune cells are able to migrate to sites of inflammation, GC are still able to suppress their function. The production of pro-inflammatory mediators and cytokines is impaired by GC. GC can limit inflammation by suppressing the production of pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα [217, 251, 252]. Phopholipase A2 and cyclooxygenase, which are required for the synthesis of prostaglandins from lipid precursors, and inducible nitric oxide synthase, an important source of NO, are also suppressed by GC treatment [253, 254]. The effects of CORT on the ability of DC to produce the pro-inflammatory cytokines IL-6 and TNFα in vitro have been addressed in Chapter III, but effects of stress on cytokine production in vivo during immune responses to HSV remain unknown.

b. Effects of stress/GC on antiviral immunity

Since stress-induced hormones can modulate the function of several types of immune cells, it is not surprising that these hormones compromise one’s resistance to viral infection. Several studies (discussed below) have found that susceptibility to various viruses is increased by stress or stress-induced GC by reducing the ability of a wide array of immune cells, including NK cells, B cells, and CD4+ T helper cells and CD8+ CTL, to confer protection from these viruses.

Restraint stress impairs the recruitment and activation of NK cells to the lung during influenza virus infection, resulting in increased amounts of viral RNA in stressed animals [255]. Several studies in humans have found that stressed individuals have weak
antibody responses to vaccination as compared to age-matched controls [256], a finding that agrees with studies in mouse models. Mice subjected to footshock stress had lower antibody titers against HSV than non-stressed control mice [8], and while restraint-stressed mice do not have decreased antibody titers, they do seroconvert more slowly in response to influenza virus infection [257].

Extensive studies have been conducted to examine the effect of stress on antiviral T cell responses. CD4\(^+\) T cell responses in mice infected with TMEV are suppressed by restraint stress, an effect that was partially-mediated by GC [6]. These T cells produce fewer cytokines and express less of the Th\(_1\)-polarizing transcription factor T-bet as compared to control mice. Treatment with exogenous GC induces CD4\(^+\) T cell responses to shift from cell-mediated Th\(_1\) responses to favor of humoral Th\(_2\) responses. This shift occurs by interrupting Th\(_1\)-promoting IL-12 signaling via GC-induced inhibition of STAT4 phosphorylation without an effect on the ability of Th\(_2\)-promoting IL-4 to signal through STAT6 [258].

i. Effects of Stress on CD8\(^+\) T cell Function – Emphasis on HSV

Stress and stress-induced GC strongly suppress CD8\(^+\) T cell responses. Most of the work examining the effects of stress-induced GC on CD8\(^+\) T cell function has focused on the CD8\(^+\) T cell response against HSV. Early studies found that stress impaired the proliferation and acquisition of cytolytic activity by CD8\(^+\) T cells in response to HSV [7]. Furthermore, the activation of memory CTL (CTLm) was impaired [259], possibly due to decreased production of cytokines [260]. The effects of stress on CTLm activation were
subsequently found to be due to hormones secreted by the adrenal glands [261] and strong enough to abrogate protection from mucosal challenge [262].

Stress can have profound consequences on the outcome of HSV infection. During intranasal infections, stress increases symptoms and mortality from HSV-induced encephalitis [263]. These effects of stress are CORT-mediated [12] and likely due to delayed infiltration of CD8$^+$ T cells into the central nervous system, which allows virus to spread, increasing collateral damage from the ensuing immune response [5]. Local CTL responses to mucosal HSV are also impaired by stress-induced CORT [264], which can result in increased viral pathogenicity in intravaginal HSV infections [265].

Following the initial infection of epithelial tissues, HSV remains latent in the sensory ganglia of the host [266]. During this latency, the viral genome is not silent but is intermittently transcribed in abortive attempts to begin viral reactivation [267]. Control of latent virus is achieved by the secretion of granzyme B by antigen-specific CD8$^+$ T cells that continuously infiltrate ganglia [182]. During psychological stress, CD8$^+$ T cell function is compromised, allowing latent HSV to reactivate from neural tissues [268].

HSV may have evolved to take advantage of suppressed CD8$^+$ T cell function by coordinating reactivation from latency with periods of stress. Molecular analyses of the HSV genome have identified a putative GRE with one of the viral origins of replication [269]. This DNA sequence is able to bind GR and facilitates DNA replication in the presence of GC. HSV-infected neuronal cells treated with DEX displayed a five-fold increase in DNA replication as compared to control cells [269]. This enhanced replication may be a method for coordinating viral reactivation with periods of stress-
induced immunosuppression. Numerous clinical reports have associated psychological stress with increased HSV reactivation in humans [270].

ii. Effects of GC on DC Function

*In vivo*, it is very difficult to distinguish between the direct effects of GC on T cells themselves and effects on the APC required for their activation. Studies that measure DC function generally assess DC function *ex vivo* or employ *in vitro*-generated DC. Until recently, nearly all studies on the effects of GC on DC function have used synthetic GC, such as DEX. The first studies to focus on the effects of GC on DC function examined murine splenic DC *in vitro*. Culturing DC in DEX decreased DC viability by 10-50% and impaired the spontaneous upregulation of B7.1 and B7.2 that occurs in cultured DC. The effects of DEX were GR-dependent and could be prevented by the addition of exogenous GM-CSF to culture media, suggesting that effects on the DC were directly related to their viability [271].

Other studies have examined the effects of GC on various aspects of DC function. When human monocyte-derived DC (MoDC) were treated with DEX during their 7 day differentiation, the resulting DC expressed lower levels of B7.2 and CD40 and were impaired for maturation in response to TNFα or CD40L [272]. DEX-treated MoDC are also less immunostimulatory and take up antigen at a higher rate than control MoDC [273]. If DC are allowed to differentiate before GC treatment, then they remain resistant to maturation-inducing stimuli. However, if differentiated DC are induced to mature, then subsequent exposure to GC has little effect [274]. While GC strongly reduced
numbers of pDC in vivo and enhanced pDC apoptosis in vitro, GC did not impair the ability of pDC to produce type I IFN in response to HSV [275].

The major caveat to the studies described above is that they often used high, often cytotoxic, doses of synthetic GC. Although this is a useful method for modeling the effects of pharmacologically administered GC on DC function, this method does not necessarily represent how endogenous GC may affect DC. Synthetic GC, such as DEX, are much more potent and have longer half-lives in vivo than naturally produced GC. Potency is determined by the ability of a specific GC to bind GR, relative to cortisol, the endogenously produced GC, which has a value of 1. DEX has a potency of 30, and a half-life that is 1.4-1.8 times that of cortisol in vivo [276]. Furthermore, synthetic GC are unable to bind CBG, and thus have a much greater bioavailability in vivo [277]. These differences between natural and synthetic GC demonstrate that these compounds may have distinct effects on immune cells both in vitro and in vivo and illustrate the need for an understanding of how naturally-produced GC affect immune cells, such as DC.

Although few studies had examined the effect of endogenously-produced GC on DC function, recent work has begun to examine the mechanisms by which stress-induced GC modulate DC function [13]. These studies used a DC-derived cell line (DC2.4) and found that direct presentation of viral antigen on MHC class I was suppressed by CORT. This impairment occurs due to a decreased efficiency of peptide generation in the cytosol, resulting in fewer MHC class I molecules complexed with viral peptide on the plasma membrane. Studies that further examine the effects of stress and stress-induced CORT on DC and their role in antiviral immunity are the subject of this dissertation.
CHAPTER III. CORTICOSTERONE IMPAIRS DENDRITIC CELL
MATURATION AND FUNCTION

The findings contained in this chapter have been previously published as Elftman MD, Norbury CC, Bonneau RH, Truckenmiller ME. Corticosterone impairs dendritic cell maturation and function. *Immunology* 2007; 122:279-290.

A. Introduction

Immature DC are highly efficient at sampling their antigenic environment but are inefficient at T-cell priming and express low levels of surface MHC class II and costimulatory molecules B7.1 and B7.2 [11, 107, 278]. Upon encounter with a pathogen or inflammatory signal, DC undergo maturation, a process that is associated with a change in function from antigen uptake to antigen presentation [114, 279]. Maturing DC up-regulate surface expression of MHC class II and costimulatory molecules [114]. Maturation eventually results in a decrease in antigen uptake [107, 118] and is accompanied by the production of pro-inflammatory cytokines [280, 281]. The net result of these changes is that mature DC possess a potent ability to prime naive T lymphocytes [11, 114, 278]. The maturation process is likely to be one of the earliest critical steps in the initiation of many adaptive immune responses.

It is well established that products and processes of the nervous and endocrine systems can have substantial effects on both innate and adaptive immunity [191, 198]. In humans and animals, external stimuli can initiate a ‘stress response’ involving activation of the HPA axis, resulting in increased production of CORT (cortisol in humans) by the adrenal glands, which is released into circulation [282]. We and others have shown that
CORT plays an important role in the stress-induced impairment of CTL-mediated antiviral immunity [9, 261, 283] leading to a high death rate from viral infection in normally resistant mice [5]. In addition to direct effects on T cells, stress-induced CORT may modulate DC function. Because DC play a crucial role in priming CTL-mediated immune responses [284, 285], it is essential to understand the role and mechanisms of neuroendocrine mediators in modulating DC function.

GC, including endogenously produced CORT, have long been known to possess immunosuppressive properties [286]. Our previous studies have shown that CORT, at physiologically relevant concentrations, impairs the generation of antigenic peptide, leading to a functional decrease in antigen processing and presentation on MHC class I by virus-infected DC [13, 287]. Here we extend these studies to examine the effects of CORT on DC maturation and function using primary BMDC. We have determined that physiologically relevant ‘stress levels’ of CORT [230, 288] acting through the GR, significantly impeded or completely blocked a number of phenotypic and functional changes associated with LPS-induced maturation of primary DC. These effects of CORT were functionally relevant, as they resulted in impaired priming of naive CD8\(^+\) T cells \textit{in vivo}. Together, these findings demonstrate that CORT impairs DC maturation and function, lending new insights into potential mechanisms underlying immunosuppression resulting from interactions of the nervous, endocrine, and immune systems. Overall, these studies further elucidate the complex role of endogenously produced, stress-associated hormones in regulating immune responses against infectious pathogens.
B. Materials and Methods

1. Mice

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed under specific pathogen-free conditions. All experiments were performed according to the guidelines of the American Association for Laboratory Animal Care International and the National Institutes of Health.

2. Generation of BMDC

BMDC were generated as described previously, with modifications [289]. Bone marrow was washed from the femurs and tibiae of mice using sterile Hanks’ balanced salt solution. Approximately $10^7$ bone marrow cells were plated in Petri dishes containing Iscove’s modified Dulbecco’s medium (IMDM) with 5% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 200 U/ml penicillin, 100 µg/ml streptomycin sulfate, 10 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF, PeproTech, Rocky Hill, NJ), and 2 ng/ml IL-4 (Sigma, St. Louis, MO). Every 2 days, cultures were gently swirled to dislodge loosely adherent cells, and half of the media was replaced with fresh media.

3. CORT/LPS treatment

Unless otherwise indicated, on day 6 of BMDC culture, at which time DC were differentiated but predominantly immature, CORT (98% pure; MP Biomedicals, Solon, OH) or vehicle (VEH, 0.1% (v/v) ethanol) was added to the culture media for 48 hr.
During the final 12 hr, cells were stimulated with 100 ng/ml *Escherichia coli* 055:B5 LPS (Sigma).

**4. GR antagonist treatment**

BMDC were generated as described above. On day 6, cells were pretreated with $10^{-6}$ M RU-486 (Sigma) for 2 hr prior to the addition of $10^{-6}$ M CORT. Thirty-six hours later, cells were stimulated with 100 ng/ml LPS for 12 hr before harvesting.

**5. Analysis of protein expression by flow cytometry**

BMDC were harvested, and Fc receptors and non-specific binding sites were blocked with supernatant from the anti-CD16/32 hybridoma (2.4G2) [290] containing 20% (v/v) mouse serum. Cells were then stained with various combinations of directly labeled antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-I-A\(^b\) (AF6-120.1; BD Biosciences, San Jose, CA), FITC-conjugated anti-CD40 (HM40-3; eBioscience, San Diego, CA), FITC-conjugated anti-B7.1 (CD80, 16-10A1; BD Biosciences), FITC-conjugated anti-B7.2 (CD86, GL1; BD Biosciences), phycoerythrin (PE)-Cy5-conjugated anti-CD11c (N418; eBioscience), and PE-conjugated anti-TLR4 (MTS510; eBioscience). In some experiments, cells were stained for surface CD11c prior to fixation with 2% paraformaldehyde and permeabilization with 0.5% (w/v) saponin (Sigma). These cells were then stained for I-A\(^b\), B7.1, or B7.2 to measure total (surface and intracellular) expression of each of these proteins. Flow cytometry was performed on a FACSCanto (Becton Dickinson, San Diego, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR). DC were identified as CD11c\(^+\) for analysis.
6. Detection of apoptosis

BMDC were harvested and stained with FITC-conjugated anti-CD11c (N418; eBioscience), PE-annexin-V (BD Biosciences), and 7-amino-actinomycin D (7-AAD; BD Biosciences). Flow cytometry was performed on a FACScan (Becton Dickinson), and data were analyzed as described above.

7. Real-time polymerase chain reaction (PCR)

The effects of CORT/LPS treatment on the transcription of genes encoding I-A\(^b\), B7.1, and B7.2 were analyzed by real-time PCR. DC were purified from BMDC cultures using magnetic CD11c microbeads with an AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. RNA was isolated using TRI reagent (Sigma), and cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, Valencia, CA). Real-time PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix (Applied Biosystems) and pre-validated TaqMan Gene Expression Assays Mm00439216_m1 (H2-Ab1), Mm00444543_m1 (B7.1), Mm00711660_m1 (B7.2), and Mm00446973_m1 (TATA-binding protein (TBP), all Applied Biosystems). Relative changes in gene expression were calculated using the \(\Delta\Delta C_T\) method, computed by the SDS software package v2.2.2 (Applied Biosystems) normalizing to TBP expression as the endogenous control.
8. Fluorescence microscopy

DC were stained for both cell-surface and intracellular antigens by indirect immunofluorescence. DC were cultured from bone marrow on glass Lab-Tek II eight-well chamber slides (Nalge Nunc International, Naperville, IL) and treated with CORT and/or LPS as described above. Non-specific binding sites were blocked with 10% (v/v) goat serum (Sigma) in Hanks’ balanced salt solution/bovine serum albumin on ice for 20 min. For co-localizing intracellular MHC class II (I-A\textsuperscript{b}) and lysosomes (lysosome-associated membrane protein (LAMP)-1), the cells were first incubated with anti-CD11c antibody clone N418 (monoclonal antibody supernatant from the hamster hybridoma) for 1–3 hr on ice. Following washes with phosphate-buffered saline (PBS), FITC-conjugated anti-hamster secondary antibody (diluted 1:200, eBioscience) was applied for 30 min. This approach allowed for visualization of surface CD11c to identify DC in the cultures. The cells were washed, fixed with 4% (w/v) paraformaldehyde, then permeabilized with 0.05% (w/v) saponin for 20 min at room temperature. Following washes, intracellular staining of the same cells was performed using a rat primary antibody against mouse LAMP-1 (CD107a, BD Pharmingen) and primary anti-mouse I-A\textsuperscript{b} (Y3P monoclonal antibody supernatant from mouse HB-183 hybridoma, American Type Culture Collection, Rockville, MD) for 1 hr at room temperature. Following washes, Alexa 546-conjugated anti-rat and Alexa 647-conjugated anti-mouse secondary antibodies (both diluted 1:200; Molecular Probes, Carlsbad, CA) were applied for 30 min. The slides were mounted with ProLong Gold antifade reagent (Molecular Probes), and examined under an Olympus IX81 deconvolution microscope with Slidebook 4.0 software. All images within a given experiment were acquired using identical exposure times.
9. Antigen uptake

The uptake of soluble protein by BMDC was determined by measuring the rates of uptake of FITC-conjugated ovalbumin (OVA, Molecular Probes). DC were purified from BMDC cultures using magnetic CD11c microbeads with an AutoMACS cell sorter according to the manufacturer’s instructions. CD11c+ cells were incubated in pre-warmed (37°C) complete phenol red-free IMDM (Invitrogen) for 30 min. FITC-OVA was added at a final concentration of 10 µg/ml. Cells were incubated at 37°C, and 3 x 10^5 cells were removed at 20 min intervals and transferred to ice-cold PBS 1% FBS. Cells were transferred to black 96-well plates (Dynex Technologies, Chantilly, VA), washed extensively, and lysed in PBS containing 0.3% (v/v) Triton-X-100. Lysates were analyzed for fluorescence using an XFluor4 Safire II plate reader (Tecan, Research Triangle Park, NC). Samples were excited at a wavelength of 490 nm, and emissions were read at 520 nm. Cells incubated with FITC-OVA on ice served as negative controls.

10. In vivo T-cell priming

DC were purified from BMDC cultures using magnetic CD11c microbeads with an AutoMACS cell sorter as described above. Cells were incubated with the immunodominant HSV-1 glycoprotein B (gB)_{498-505} peptide (100 nM, SSIEFARL) for 40 min, washed and resuspended in PBS containing 1% (v/v) FBS. Mice received 5 x 10^5 CORT-treated or VEH-treated cells intravenously in a volume of 500 µl. One week later, the mice were euthanized and their spleens were removed. Spleens were homogenized by passage through 60-gauge stainless steel mesh screens. The resulting cell suspension
was purified using Lymphocyte Separation Media (Cambrex Bio Science, Walkersville, MD). Cells were then used for intracellular cytokine staining or degranulation assays.

11. Intracellular cytokine staining

Splenocytes were incubated in 96-well plates (4 x 10⁶ cells/well) in the presence of 10 μM HSV-1 gB₄₉₈-₅₀₅ or an irrelevant peptide (OVA₂₅₇-₂₆₄) at 37°C. After 2 hr, 5 μg/ml brefeldin A (Sigma) was added to the cells. After an additional 4 hr, the cells were stained with PE-Cy5-conjugated anti-CD8α (53–67; eBioscience). Cells were then fixed with 2% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) saponin, and stained intracellularly with FITC conjugated anti-IFNγ (XMG1.2; eBioscience) and PE-conjugated anti-TNFα (MP6-XT22; eBioscience).

BMDC were treated with CORT and/or LPS as described above. Four hours prior to harvesting, the cells were treated with 5 μg/ml brefeldin A to inhibit cytokine secretion. BMDC were harvested and stained with FITC-conjugated anti-CD11c. Cells were fixed with 2% (w/v) paraformaldehyde, permeabilized with 0.5% (w/v) saponin, and stained intracellularly with PE-conjugated anti-IL-6 (MP5–20F3; eBioscience), PE-conjugated anti-IL-12 p40 (C17.8; eBioscience), or PE-conjugated anti-TNFα. Flow cytometry was performed on splenocytes and BMDC as described above.

12. Degranulation assay

The regulated secretion of lytic granules from CTL is triggered by T-cell receptor recognition of a target cell. CD107a (LAMP-1) is found on lysosomal membranes. These membranes are transiently exposed to the extracellular media during CTL
degranulation. By incubating cells with antibody against CD107a, we were able to measure degranulation in response to a specific peptide. Splenocytes were incubated in 96-well plates (4 x 10^6 cells/well) in the presence of 10 µM HSV-1 gB_{498-505} or an irrelevant peptide (OVA_{257-264}) at 37°C. Wells also contained FITC-conjugated anti-CD107a (1D4B; BD Pharmingen). After 1 hr, 10 mM NH₄Cl was added to cells to prevent endosome acidification. Three hours later, the cells were stained with PE-conjugated anti-I-A^b (AF6-120 1; BD Pharmingen) and PE-Cy5-conjugated anti-CD8α. Flow cytometry was performed using a FACSCanto, and data were analyzed using FlowJo software. Cells were stained for I-A^b to exclude CD8α^+ DC that constitutively undergo endocytosis and take up anti-CD107a. Analyses were performed on the CD8^+, I-A^b_{dim} population of cells.

13. Statistical analyses

Statistical analyses were performed by applying Student’s t-test. Significance was determined as p < 0.05. Error bars in all figures represent standard error of the mean.
C. Results

1. Generation of BMDC

Bone marrow-derived cells were cultured in the presence of GM-CSF and IL-4 for 8 days. At the end of this time, the cultures predominantly contained DC (80–90%) that were CD11c\(^+\), CD11b\(^+\), CD8\(\alpha\)^\(-\), resembling myeloid DC described in vivo [291, 292]. Most of these cells (90%) were immature, expressing low levels of MHC class II, B7.1, and B7.2 as measured by mean fluorescence intensity (MFI) of antibody staining (Fig. 2a, shaded histogram).

2. CORT impairs LPS-induced up-regulation of maturation-associated markers

DC maturation is associated with increased surface expression of MHC class II and the costimulatory molecules, B7.1, B7.2, and CD40. To determine the effects of CORT on DC maturation, we treated BMDC with CORT (10\(^{-6}\) M) or VEH for 48 hr. This concentration of CORT is similar to endogenous concentrations that we and others have measured in mice undergoing a stress response (1-3 x 10\(^{-6}\) M) [230, 288]. During the final 12 hr of CORT treatment, cells were also treated with LPS (100 ng/ml) to induce maturation. After LPS stimulation, the cells were harvested and stained for CD11c, MHC class II, B7.1, B7.2, and CD40 for analysis by flow cytometry. On CD11c\(^+\) cells, MHC class II, B7.1, B7.2, and CD40 each showed substantial increases in surface expression after LPS stimulation (averaging 46\%, 90\%, 102\%, and 194\% increases in MFI, respectively, Fig. 2b, c). The effects of LPS on MHC class II and B7.2 expression were completely blocked when the cells were pretreated with CORT, while the effect on B7.1 was reduced by 50\%, and CD40 upregulation was only slightly attenuated (Fig. 2b,
Figure 2. Effects of CORT/LPS on DC maturation-associated marker surface expression. (a) Representative histograms depicting the surface staining profile for VEH (shaded) and CORT-treated (dashed line) DC for the expression of each marker. CD11c⁺ cells were gated for analysis. (b) Representative histograms depicting the surface staining profile for VEH-treated (shaded), LPS-treated (thin line), and CORT/LPS treated (bold line) DC for the expression of each marker. CD11c⁺ cells were gated for analysis. (c) Surface expression of maturation-associated markers was determined by flow cytometry. MFI were normalized to VEH-treated cells (100% baseline MFI) for seven separate experiments. * = p < 0.05.
Figure 2

(a) % of Max

(b) % of Max

(c) % Baseline MFI

- **CORT**
- **LPS**
- **CORT/LPS**

MHC class II  B7.1  B7.2  CD40

VEH  VEH  VEH  VEH
c). These results suggest that CORT impairs DC maturation. Although CORT treatment alone slightly reduced the expression of each marker, this treatment did not result in a substantial change of DC staining profiles (Fig. 2a), because unstimulated cells were mostly immature. Time-course studies demonstrated that cells required 12 hr of exposure to CORT to maximally impair DC maturation. CORT treatment of DC for 6 hr or less failed to substantially affect LPS-induced expression of MHC class II, B7.1, or B7.2.

3. DC maturation is impaired by physiological concentrations of CORT

CORT is constitutively produced in vivo at low levels but is up-regulated under conditions of psychological stress. At higher concentrations, CORT mediates its effects by binding the GR. Because DC maturation was impaired by 10^{-6} M CORT, we examined the effects of a range of concentrations of CORT (10^{-9}-10^{-6} M) on DC maturation. A low dose of CORT (10^{-9} M), as compared to no CORT (VEH), slightly enhanced the effect of LPS on BMDC. However, concentrations of CORT > 10^{-8} M, saturating the GR (GR K_d = 0.5-1 x 10^{-8} M), significantly impaired or blocked the LPS-induced up-regulation of MHC class II (Fig. 3a), B7.1 (Fig. 3b), and B7.2 (Fig. 3c).

4. Effects of CORT are mediated through the GR and are not caused by apoptosis

To determine whether the effects of CORT on DC maturation were mediated through the GR, we used the GR antagonist RU-486. BMDC were pretreated with RU-486 for 2 hr prior to treatment with CORT and/or LPS. While RU-486 alone slightly reduced the LPS-induced up-regulation of MHC class II, B7.1, and B7.2, the GR antagonist completely prevented CORT from having any additional effects on the LPS-
Figure 3. CORT acts at concentrations that saturate the GR and is blocked by pre-treatment with GR antagonist.  (a-c) Dose-response relationship between CORT and surface expression of maturation-associated markers. DC were treated with various concentrations of CORT for 48 hr and stimulated with LPS during the last 12 hr. Surface expression was analyzed by flow cytometry for (a) MHC class II, (b) B7.1, and (c) B7.2. Normalized MFI relative to VEH-treated cells without LPS are displayed for the average of three separate experiments. * = p < 0.05.  (d) GR specificity of CORT. DC were pretreated with RU-486 for 2 hr before subsequent treatment with CORT and/or LPS. Surface expression of maturation-associated markers was analyzed by flow cytometry. Data are expressed as MFI normalized from three separate experiments.
Figure 3

(a) MHC class II

(b) B7.1

(c) B7.2

(d) Comparison of different treatments:

- LPS
- RU-486/LPS
- CORT/LPS
- RU-486/CORT/LPS

Legend:

- MHC class II
- B7.1
- B7.2

Cort (M) vs LPS: 0 0 10^{-9} 10^{-8} 10^{-7} 10^{-6}

VEH VEH VEH
induced up-regulation of these markers (Fig. 3d). These results suggest that the impairment of DC maturation by CORT is mediated through the GR.

GC have been reported to induce apoptosis in DC under certain conditions [293]. It was possible that CORT had induced apoptosis in DC that otherwise would have undergone maturation. To address this issue, we stained CORT/LPS-treated BMDC with annexin-V and 7-AAD to detect early apoptotic and dead cells. However, in three separate experiments, CORT did not increase the percentage of early apoptotic (annexin-V\(^+\), 7-AAD\(^-\)) or apoptotic/dead (annexin-V\(^+\), 7-AAD\(^+\)) cells over the 48 hr treatment period examined (Fig. 4a), suggesting that the observed CORT-induced impairment of DC maturation was not caused by increased apoptosis or cell death.

Because LPS induces DC maturation through binding to TLR4, [19] it was possible that CORT reduced the surface expression of TLR4, which would make DC resistant to LPS-induced maturation. However, we did not observe any reduction in surface TLR4 expression in CORT-treated DC. Furthermore, LPS-induced TLR4 downregulation was not modulated by CORT (Fig. 4b).

5. CORT impairs transcription of B7.1 and B7.2 and causes intracellular retention of MHC class II

To determine the mechanism by which CORT impaired the up-regulation of DC maturation markers, we examined mRNA and total protein levels of MHC class II, B7.1, and B7.2 in CORT/LPS-treated DC. Total protein expression (surface and intracellular) for each of these molecules was determined by staining permeabilized cells for flow cytometry. Staining profiles for total B7.1 and B7.2 (Fig. 5a, b) were similar to those
Figure 4. CORT does not induce apoptosis or downregulate TLR4 on DC. (a) Scatterplots depicting Annexin V/7-AAD staining of CD11c+ cells treated with CORT or VEH. Scatterplots are representative of results from three separate experiments. (b) Surface expression of TLR4 was determined by flow cytometry. MFI were normalized to VEH-treated cells (100% baseline MFI) for three separate experiments.
Figure 4

(a) Scatter plots showing the relationship between Annexin V and 7-AAD for VEH and CORT treatments.

(b) Bar graph showing TLR4 expression (% Baseline MFI) for VEH, CORT, LPS, and CORT + LPS conditions.
**Figure 5.** Effects of CORT/LPS on protein and mRNA expression of DC maturation-associated markers. CORT/LPS treated cells were fixed and permeabilized prior to staining for flow cytometry. (a) Representative histograms depicting the total protein staining profile for VEH-treated (shaded) and CORT-treated (dashed line) DC for the expression of each marker. (b) Representative histograms depicting the total protein staining profile for VEH-treated (shaded), LPS-treated (thin line), and CORT/LPS treated (bold line) DC for the expression of each marker. (c) MFI were normalized to VEH-treated cells for three separate experiments. (d) Relative mRNA expression of each gene as determined by real-time RT-PCR. Data represent the mean of three separate experiments. * = $p < 0.05$. 
obtained from surface-stained cells (Fig. 2a, b). LPS-treated DC contained significantly more B7.1 and B7.2 than VEH-treated cells (average of 2.0- and 2.4-fold increases, respectively), and these increases were partially (B7.1) or completely (B7.2) inhibited in cells that were pretreated with CORT (Fig. 5b, c).

Quantitative real-time PCR was used to measure the transcripts encoding these proteins. LPS treatment resulted in significantly increased transcription of B7.1 and B7.2 (average of 4.3- and 3.8-fold, respectively, Fig. 5d). Furthermore, CORT-treatment significantly impaired this increase, with a greater effect on B7.2 than on B7.1. These results correlate well with our observations that CORT impairs the LPS-induced expression of B7.1 and B7.2 protein (Figs. 2c and 5c), demonstrating that CORT modulated the expression of B7.1 and B7.2 through effects on the transcription or stability of RNA.

Although total protein staining for B7.1 and B7.2 resulted in expression profiles similar to those of non-permeabilized cells, intracellular staining of MHC class II did not resemble MHC class II surface expression. Recall that surface expression of MHC class II was low in VEH-treated cells and increased upon LPS-stimulation, but remained low when cells were pretreated with CORT (Fig. 2b). Permeabilized DC stained strongly for MHC class II, regardless of whether they were treated with CORT and/or LPS (Fig. 5a-c). When MHC class II transcripts were measured, the amounts of mRNA did not change in DC that had been treated with CORT and/or LPS (Fig. 5d). These data indicate that CORT did not have substantial effects on MHC class II at the transcriptional or translational levels, indicating that the effect of CORT on MHC class II was post-translational.
Besides transcriptional and translational regulation, cell surface proteins can be regulated at the level of trafficking. The localization of MHC class II in CORT/LPS-treated cells was examined by deconvolution microscopy (Fig. 6). In VEH- and CORT-treated cells, MHC class II was retained intracellularly and colocalized with the lysosomal marker LAMP-1. Upon LPS treatment, MHC class II was expressed predominantly at the cell surface. However, when cells were treated with CORT prior to LPS stimulation, MHC class II was retained intracellularly and colocalized with LAMP-1. These studies indicate that the effects of CORT on surface expression of MHC class II are mediated by inhibiting LPS-induced trafficking to the plasma membrane.

6. CORT prevents LPS-induced down-regulation of endocytosis

The above studies show that CORT impaired the surface expression of maturation-associated phenotypic markers, including the LPS-induced up-regulation of MHC class II, B7.1 and B7.2. Next, we determined the effects of CORT on DC function, beginning with antigen uptake. Immature DC continuously sample their antigenic environment. Upon encounter with a maturation-inducing stimulus, DC transiently increase their rate of antigen uptake before strongly down-regulating endocytosis (3,6). Rates of uptake of soluble protein (FITC-OVA) were measured in DC that had been treated with CORT and/or LPS. As expected, LPS treatment significantly reduced (47 ± 6%) the rate of antigen uptake compared to VEH-treated cells (Fig. 7). However, cells that were treated with CORT prior to LPS-stimulation endocytosed antigen at a rate similar to VEH-treated cells. Cells treated with CORT alone were also similar to VEH-
Figure 6. Effects of CORT/LPS on cellular localization of MHC class II. DC cultures were treated with CORT and/or LPS as described and stained for surface CD11c (green), intracellular LAMP-1 (red), and MHC class II (blue). Deconvolution microscopy was used to visualize cells. The images in this figure were acquired by Dr. Emmy Truckenmiller.
<table>
<thead>
<tr>
<th></th>
<th>LAMP-1</th>
<th>MHC II</th>
<th>CD11c</th>
<th>Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEH</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td><strong>CORT</strong></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td><strong>CORT/LPS</strong></td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
</tr>
</tbody>
</table>

Scale bar: 25 μm
Figure 7. Effect of CORT/LPS on endocytosis of soluble protein. (a) DC were treated with CORT/LPS and incubated with FITC-OVA at 0°C (dashed lines) or 37°C (solid lines). Aliquots were removed at 20 min intervals, washed extensively, and lysed. FITC-OVA uptake was measured by fluorimetry. Data from a representative time-course are shown, expressed in relative fluorescence units (RFU). (b) The average rates of uptake from three separate experiments were normalized to VEH-treated cells. * = p < 0.05.
Figure 7

(a) Fluorescence (RFU) over time in minutes. The graph shows the fluorescence levels for VEH, CORT, LPS, and CORT/LPS treatments. 

(b) % Uptake Rate (rel. VEH) comparing VEH, CORT, LPS, and CORT/LPS. The inset graph illustrates significant differences indicated by asterisks (*).
treated cells. This assay specifically measured active uptake, as control cells incubated on ice did not take up FITC-OVA (Fig. 7a).

7. CORT impairs cytokine production by DC

Activated DC produce cytokines that regulate the initiation of immune responses [121, 294]. We determined the effects of CORT on LPS-induced cytokine production by DC using intracellular cytokine staining. LPS stimulation induced the synthesis of IL-6, IL-12, and TNFα. CORT treatment significantly reduced the percentage of LPS-stimulated cells that produced IL-6 (29 ± 2% versus 45 ± 3%) and IL-12 (21 ± 1% versus 46 ± 2%) (Fig. 8a). In addition, CORT/LPS-treated cells produced significantly less IL-6, IL-12, and TNFα (averaging 55 ± 0.4%, 58 ± 4%, and 74 ± 3% decreases in the MFI of cytokine-positive cells, respectively), on a per cell basis than cells treated with LPS alone (Fig. 8b).

8. CORT renders DC inefficient at priming CD8⁺ T-cell responses

One of the most critical functions of DC is their role in priming T cells to mount an immune response against viral infection. We determined the effects of CORT on the ability of DC to prime CD8⁺ T-cell responses in vivo by measuring peptide-specific CD8⁺ T-cell responses. The use of an adoptive transfer strategy enabled us to separate the effects of CORT on the DC from the T cells in vivo. Mice were injected intravenously with DC pulsed with the HSV-1 gB₄₉₈-₅₀₅ peptide, resulting in an antigen-specific CD8⁺ T-cell response as measured by intracellular cytokine staining for IFNγ and TNFα. CTL-mediated cytotoxicity was also assessed by measuring degranulation in response to
**Figure 8.** Effect of CORT/LPS on cytokine production by DC. Prior to harvesting, DC were treated with CORT/LPS, as described, and brefeldin A was added for 4 hr to inhibit cytokine secretion. Cells were permeabilized and stained for IL-6, IL-12, and TNFα. (a) Percentages of cells containing each cytokine are displayed. Data were averaged from three separate experiments. (b) Relative amounts of cytokine produced on a per cell basis in cytokine-containing cells are displayed. Data were averaged from three separate experiments and normalized to LPS-treated cells (100%). * = p < 0.05.
Figure 8

(a) % Cytokine+ Cells (CD11c+)

- VEH
- CORT
- LPS
- CORT/LPS

(b) % MFI (rel. LPS)

- IL-6
- IL-12
- TNF-α

LPS
CORT/LPS

* indicates statistical significance.
specific peptide. Degranulation of CTL is triggered upon T-cell receptor recognition of a target cell. However, when mice received DC that were treated with CORT prior to peptide-pulsing, the resulting peptide-specific CD8⁺ T-cell responses were reduced significantly, as measured by IFNγ (Fig. 9a), TNFα (Fig. 9b), and degranulation (Fig. 9c). These responses were antigen-specific, as they did not occur when splenocytes were pulsed with an irrelevant peptide (OVA_{257-264}). Mice injected with DC in the absence of peptide resulted in responses similar to naive mice (Fig. 10).
Figure 9. Effect of CORT on the ability of DC to prime T cell responses in vivo. CORT or VEH-treated DC were pulsed with HSV-1 gB\textsubscript{498-505} peptide, washed, and injected into mice i.v. Seven days later, splenocytes were isolated and assayed for production of (a) IFN\(\gamma\), and (b) TNF\(\alpha\), or (c) degranulation in response to HSV gB\textsubscript{498-505} or OVA\textsubscript{257-264} (irrelevant peptide). Splenocytes from naive mice were used as negative controls. Data represent the mean responses from groups of 4-6 mice. * = \(p < 0.05\).
Figure 9

(a) % IFN-γ⁺ Cells (CD8⁺)

(b) % TNF-α⁺ Cells (CD8⁺)

(c) % CD107α⁺ Cells (CD8⁺, H-2Kb)
**Figure 10.** Injection of empty DC alone does not induce a CD8$^+$ T cell response. (A, C, E) Mice received $5 \times 10^5$ LPS-treated DC without peptide i.v. or $10^7$ PFU HSV-1 i.p. Seven days later, splenocytes were isolated and assayed for (A) degranulation and production of (C) IFN$\gamma$ and (E) TNF$\alpha$ in response to HSV gB$_{498-505}$ or OVA$_{257-264}$ (irrelevant peptide). (B, D, F) In a separate experiment, CD8$^+$ T function in spleens of mice infected 7 d previously was compared to CD8$^+$ T cell function in spleens of naive mice by peptide-specific (B) degranulation, and the production of (D) IFN$\gamma$ and (F) TNF$\alpha$. Data represent the mean responses from groups of 2 mice.
Figure 10

A  B
% CD107⁺ Cells (CD8⁺, I-Ab⁻)

0  2  4  6  8  10  12  14

C  D
% IFN-⁺ Cells

0  2  4  6  8  10  12

E  F
% TNF-⁺ Cells (CD8⁺)

0  0.2  0.4  0.6  0.8  1  1.2  1.4  1.6  1.8  2

DC alone  HSV-1

Naive  HSV-1

- gB
- Irrelevant
D. Discussion

Many studies have demonstrated that animals undergoing a stress response exhibit reduced CTL function [9, 261, 283], which can lead to a high death rate following viral infection in normally resistant mice [5]. An important component of this response involves the activation of the HPA axis resulting in the production of CORT that, in addition to directly impairing T lymphocyte function, may interfere with the ability of DC to prime naive T cells. Most of the studies examining the effects of GC on DC function have used the synthetic pharmacological GC, DEX [271-274, 295]. In our previous work [13, 287] and current studies, we used the naturally produced GC, CORT, which is more appropriate for examining the effects of neuroendocrine interactions on immune function.

DC maturation is important for efficient priming of naive T lymphocytes [296] and has been shown to be critical in the initiation of an adaptive immune response against viral infection [297]. In our studies, the CORT-mediated impairment of DC maturation was associated with a deficiency in priming antigen-specific CD8$^+$ T-cell responses (Fig. 9). Mice receiving CORT-treated DC generated fewer cytokine-producing CD8$^+$ T cells and fewer degranulating cytotoxic CD8$^+$ T cells than mice receiving VEH-treated DC. These data suggest that elevated CORT concentrations found in vivo during a stress-response likely contribute to the inefficient generation of CTL-mediated immunity. A delay or failure to efficiently prime CTL can compromise the successful control of an infection [5].

Here, we have demonstrated that CORT-treated DC were deficient in the up-regulation of the costimulatory molecules, B7.1 and B7.2 (Fig. 2). Immature DC express
low levels of costimulatory molecules, making them inefficient for T-cell priming [296]. Upon maturation, DC normally up-regulate B7.1 and B7.2 to provide the necessary costimulatory signal to naive T cells through CD28. A lack of costimulation can lead to T-cell anergy [111] and could contribute to our observed deficiency in T-cell priming in mice that received CORT-treated DC.

The up-regulation of costimulatory molecules alone is insufficient for T-cell priming [298]. Mature DC also secrete many cytokines, including IL-6, IL-12, and TNFα. IL-6 and TNFα are generally proinflammatory, and IL-6 renders effector T cells resistant to suppression by T_{Reg} cells [299]. IL-12 is important for promoting cell-mediated T_{H1} polarization in CD4^{+} T cells [300]. We observed that fewer CORT-treated DC were able to produce IL-6 and IL-12 in response to stimulation with LPS, and that CORT reduced the amount of IL-6, IL-12, and TNFα on a per cell basis (Fig. 8). The observed reduction in T-cell priming in vivo could be caused by reduced costimulatory molecule expression, cytokine production, or a combination of these factors. In addition, it is possible that these effects could also interfere with priming of CD4^{+} T cells. Others have previously reported that mice undergoing a stress response exhibit strong T_{H2} polarization [301, 302]. These effects could be the result, in part, of reduced production of IL-12 or other cytokines by DC.

The effect of CORT on antigen uptake is further evidence that CORT interferes with DC maturation. While immature DC are efficient at antigen uptake, LPS-stimulation transiently increases endocytosis before strongly inhibiting it [118]. This increase is thought to ‘load up’ the DC with the antigen in its immediate environment prior to migration to a LN for presentation to T cells. We observed that antigen uptake
was reduced after 12 hr of LPS stimulation, as expected. However, CORT treatment prevented this down-regulation (Fig. 7). Although antigen uptake remained high in CORT-treated DC, our previous findings have shown that antigen processing and presentation are impaired in CORT-treated DC [13]. Together, these results have significant implications for the effect of CORT on cross-presentation, suggesting that the rate of antigen uptake may not be able to compensate for the effect of CORT on antigen processing and presentation. The effects of CORT and stress on cross-presentation, both in vitro and in vivo, are currently being studied in our laboratory.

Our data suggest that the mechanism of action of CORT is to directly oppose LPS as a DC maturation-inducing stimulus. In LPS-stimulated DC, transcription and expression of B7.1 and B7.2 are increased, while preformed MHC class II is transported from intracellular vesicles to the plasma membrane without any changes in MHC class II transcription [108]. CORT treatment impaired both transcription and protein expression of B7.1 and B7.2 in LPS-stimulated cells (Fig. 5). In contrast, surface MHC class II expression is reduced in CORT-treated cells via retention of pre-existing MHC class II molecules within intracellular compartments (Fig. 6). These results demonstrate that CORT rendered DC resistant to the maturation-inducing effects of LPS. However, the surface expression of TLR4 did not decrease upon CORT treatment, indicating CORT was not simply reducing the ability of DC to detect LPS. Furthermore, we found that CORT also prevented DC maturation induced by poly (I:C) or CpG DNA (Fig. 11), which induce maturation through TLR3 and TLR9, respectively. These findings suggest that the effects of CORT are not limited to LPS-induced maturation. Because DC maturation and cytokine production are mediated by distinct pathways downstream of
Figure 11. Effect of CORT on CpG DNA or poly (I:C)-induced DC maturation-associated marker surface expression. BMDC were generated and treated with CORT or VEH for 36 hr. CpG oligodeoxynucleotide 1826 (100 nM) or poly (I:C) (50 µg/ml) were added for the final 12 hr of culture. Flow cytometric analysis was performed as described above. Histograms depict the surface staining profile for VEH-treated (shaded), TLR agonist-treated (thin line), and CORT/TLR agonist-treated (bold line) DC for the expression of each marker. CD11c^+ cells were gated for analysis.
Figure 11

CpG

Poly (I:C)

CD11c

MHC Class II

B7.2

% of Max

% of Max

% of Max

% of Max
TLR4 [303] and both were modulated by CORT, it is likely that CORT acts on multiple intracellular targets in DC. Because the effect of CORT on DC maturation was blocked by a GR antagonist (Fig. 3d), it is also likely these effects are mediated via the GR.

We have shown that CORT, a physiologically produced GC, functionally impaired DC maturation and cytokine production and reduced the ability of DC to prime naive CD8+ T cells \textit{in vivo}. This inhibition occurred via the GR with concentrations of CORT similar to those observed in animals undergoing a stress response [230]. The deficiencies in DC function we have observed represent a potential mechanism underlying the inefficient generation of antiviral CTL responses observed in stress-associated immunosuppression. These findings illustrate the substantial effect that products of the nervous and endocrine systems have on immune function, and underscore the importance of considering neuroendocrine processes that can influence the outcome of an immune response.
CHAPTER IV. CHRONIC STRESS IMPAIRS ANTIVIRAL CD8$^+$ T CELL RESPONSES VIA EFFECTS ON DENDRITIC CELLS, RESULTING IN INCREASED VIRAL PATHOGENESIS

A. Introduction

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 of the HPA axis by the perception of stress. This initiates a hormonal cascade resulting in the systemic release of GC that bind to GR present in all nucleated cells. Short-term exposure to stress (applied for seconds to a few hours) can be immunostimulatory [200]. However, it has long been both anecdotally and empirically recognized that prolonged systemic elevation of stress-induced GC is immunosuppressive and can lead to deterioration of health. Chronic stress has been associated with an increased incidence of heart disease and cancer, decreased resistance to infections, and poor responses to vaccines [2, 191, 198, 304].

The primary strategy employed by the immune system in response to intracellular pathogens, some tumors and vaccines occurs via cytotoxic CD8$^+$ T cell activity. Suppression of CD8$^+$ T cell-mediated immunity can have severe consequences for the health of the host. Through efforts to understand the mechanisms underlying stress-induced immunosuppression, it has been well documented that T cell responses against a variety of viruses are compromised by GC hormones, either stress-induced (CORT or cortisol in humans) or pharmacologically administered (DEX or other synthetic GC) [6, 14, 264, 305, 306]. Stress-induced GC have been shown to substantially impair CD8$^+$ T
cell activation, proliferation, cytokine production, trafficking, cytotoxicity, and control of viral replication [5-7, 9]. Indeed, stress during infection and the resulting changes in T cell responses have lead to profound increases in mortality from influenza, HSV, and TMEV infections [5, 14, 307]).

Implied in studies of stress-induced immunosuppression is that stress-induced GC act directly on the T cells to impair T cell function. However, antiviral CD8+ T cell activation can occur only through instructions supplied to them by antigen presenting cells, in particular DC. DC are thought to be the most important type of APC in the mammalian immune system and act as the bridge between innate and adaptive immunity [11]. While the consequences of prolonged elevations of stress-induced GC are well known, the underlying mechanisms leading to these outcomes, and the role of DC in particular, have only begun to be examined in detail.

Previous studies have shown that the synthetic GC, DEX, suppresses the generation, maturation, and immunostimulatory properties of DC [271, 272, 274, 308]. We have reported that physiologically relevant “stress levels” of naturally occurring CORT inhibit TLR agonist (LPS, CpG and poly-I:C)-induced maturation of DC in a GR-dependent manner (see Chapter III). These effects include reduced intracellular MHC class II trafficking, co-stimulatory molecule expression, pro-inflammatory cytokine production, and the in vivo priming of an HSV-gB-specific CD8+ T cell response in a DC vaccination model. We also demonstrated that relevant stress levels of CORT, in a GR-dependent manner, suppress the efficiency of presentation of antigen-specific peptide-MHC class I complexes by virus-infected DC via a mechanism involving the decrease in the rate of production of antigenic peptides [13]. These previous studies describe the
molecular mechanisms by which stress-induced GC suppress DC function and their ability to activate CD8$^+$ T cells.

In the studies described in this chapter, we have identified the earliest targets of GC and determined the consequences of exposure to stress early during an \textit{in vivo} HSV infection. We used a model in which stress was applied only early during a cutaneous HSV infection and terminated before T cell effector function was detectable. This strategy allowed us to delineate the early effects of stress on the CD8$^+$ T cell response against HSV and to follow the course of infection and viral pathology after stress was terminated. Previously, it has not been feasible to identify specific cellular targets of stress-induced GC \textit{in vivo}. We accomplished this by using GR-T$_{KO}$ mice lacking GR only on their T cells [309]. We report here that: (i) the course and pathology of a cutaneous HSV infection is exacerbated when stress was experienced at the initial stage of infection; (ii) even after stress was stopped, subsequent HSV-specific CD8$^+$ T cell responses were still reduced; (iii) using the GR-T$_{KO}$ mice, the impaired T cell responses and control of viral replication \textit{in vivo} were not due to effects of stress/GC on the T cells; (iv) instead, the DC from draining LN of infected, stressed mice were significantly impaired in their ability to prime HSV gB-specific CD8$^+$ T cells. Our findings demonstrate the vulnerability of DC to stress/CORT at the earliest stages of a viral infection, having consequences for CD8$^+$ T cells and the course of infection that persist after stress has been terminated.
B. Materials and Methods

1. Mice

Male C57BL/6 mice (Jackson Laboratories or National Cancer Institute, Frederick, MD) were housed under specific pathogen-free conditions. gB-T-1.3 TCR-transgenic mice are specific for the immunodominant HSV-1 epitope, gB_{498-505} and were a provided by Dr. F. Carbone (University of Melbourne, Melbourne, Australia) [310].

GR-T_{KO} mice have a portion of Nr3c1, the gene encoding GR, flanked by LoxP sites. Cre recombinase is expressed under the Lck promoter, ablating GR expression in T lymphocytes of Cre^{+} mice [309]. GR-T_{KO} mice were provided by Dr. L. Muglia (Washington University, St. Louis, MO). All animals were maintained in microisolator cages on a 12hr/12hr light/dark cycle and treated in accordance with standards of the National Institutes of Health and American Association of Laboratory Animal Care. All animal-related procedures were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

2. Restraint stress

Using a well established restraint stress procedure (Rev. in [234]) 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. Mice were restrained for up to 4 daily sessions, beginning 1 d prior to infection. Restraint was for 16 h beginning 3 h into the dark cycle. Food and water were withheld from age-matched control mice during this time. This method for restraint stress has
been shown to increase circulating levels of the GC, CORT, to between 5 and 10-fold from baseline levels without causing physical injury [238, 288].

3. HSV infection

Mice were anesthetized by an i.p. injection of sodium pentobarbital and infected with 3 \( \times 10^5 \) PFU HSV-1 Patton in a volume of 4 µl of PBS containing 1% FBS in each hind footpad via the multiple piercing method [174]. Virus was applied to the footpad and abraded into the skin by approximately 60 punctures with a 27 gauge needle.

4. Exogenous CORT administration

In some experiments, mice were provided CORT (150 µg/ml, MP Biomedicals) or VEH (0.6% 2-hydroxypropyl-β-cyclodextrin, Sigma) in tap water, ad libitum, for one day prior to and three days following HSV-infection.

5. GR antagonist administration

In some experiments, mice were treated with the GR antagonist, RU-486 (625 µg/mouse, Sigma) [238, 264]. RU-486 was dissolved in sesame oil (MP Biomedical) and administered by subcutaneous injection 24 hr prior to the first stress session of stress and daily 2 hr prior to the initiation of each stress session.

6. HSV lesion scoring

The hind footpads of restraint-stressed or control mice were photographed daily using a digital camera. Images were scrambled and blindly scored for HSV lesion
severity on a score of 0 (no lesion), 1 (covering less than 25% of footpad), 2 (covering 25-50% of footpad), or 3 (covering more than 50% of footpad).

7. Quantification of infectious virus

Hind footpads were collected at various times post-infection and homogenized using ground-glass homogenizers. Viral load was measured from homogenates by standard plaque assay on Vero cells. Cells were then fixed and stained with a solution of 5% (v/v) formaldehyde/0.5% (w/v) crystal violet. Plaques were counted and normalized to the volume of homogenate obtained for each footpad.

8. Quantitative PCR

DNA was isolated from popliteal LN (PLN) of HSV-infected mice using the QIAamp DNA Isolation Kit (Qiagen) according to the manufacturer’s instructions. Real-time quantitative PCR was performed using Quantitect SYBR Green PCR Kit (Qiagen), primers directed at amplifying a portion of the HSV-1 viral genome encoding gB, and the ABI 7900HT Sequence Detection System (Applied Biosystems). Primer sequences were 5’-GCAGTTTACGTACAACCACATACAG-3’ and 5’-AGCTTGCAGGAGTCCCTCGT-3’ [311].

9. Western blotting

Enriched cells were obtained using Pan-DC or Thy-1.2 microbeads on an AutoMACS magnetic cell sorter (Miltenyi Biotec). DC from spleens, Thy-1.2\(^+\) cells from thymi, or non-enriched cells from the whole thymus were lysed in SDS sample
buffer. Protein from 5 x 10⁵ cells was loaded into NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) for electrophoresis and transferred to nitrocellulose membranes. Membranes were probed for GR (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Rockland, Gilbertsville, PA) to control for loading. Proteins were detected using peroxidase-anti-rabbit F(ab)₂ fragment (Jackson ImmunoResearch, West Grove, PA) and enhanced chemiluminescence reagents (Pierce, Rockford, IL).

10. Detection of antigen-specific CD8⁺ T cells

PLN were dissociated and a single-cell suspension was obtained. Fc receptors were blocked using 2.4G2 hybridoma supernatant [290] containing 20% mouse serum. Cells were stained with peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD8α (53-6.7; BD Biosciences) and PE-labeled Kᵇ-HSV gB₄₉₈-₅₀₅ tetramer (NIH Tetramer Facility, Atlanta, GA) to identify HSV-specific CD8⁺ T cells. Flow cytometry was performed using a FACSCanto (Becton Dickenson) and analyzed using FlowJo software (TreeStar).

11. Intracellular cytokine staining

PLN were dissociated into single-cell suspensions and cells were incubated with 1 µM gB₄₉₈-₅₀₅ (SSIEFARL) or OVA₂₅₇-₂₆₄ (SIINFEKL) peptide. After 2 hr, 5 µg/ml brefeldin A (Sigma) was added to prevent cytokine secretion. Four hours later, cells were stained for CD8α, as described above. After surface staining, cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% (w/v) saponin and stained with FITC-
conjugated anti IFNγ (XMG1.2; eBioscience) and Alexa 647-conjugated anti-human Granzyme B (GB11; BD Biosciences).

12. Degranulation assay

PLN were dissociated into single-cell suspensions and cells were incubated with 1 μM gB_{498-505} or OVA_{257-264} peptide in media containing FITC-conjugated anti-CD107a (1D4B; BD Biosciences). After 1 hr, 10 mM NH₄Cl was added to prevent endosome acidification. Three hours later, cells were stained for CD8α and I-A^b (AF6-120.1, BD Biosciences) as described above.

13. Proliferation assay

PLN cells were collected from mice 24 hr after HSV infection. DC were enriched by magnetic separation using Pan DC Microbeads and an AutoMACS cell sorter (Miltenyi Biotec). gB-specific CD8^+ T cells were enriched from the spleens of naïve gB-T TCR transgenic mice using CD8 microbeads (Miltenyi Biotec). Enriched CD8^+ T cells were labeled with carboxyfluorescein diacetate, succinimidy l ester (CFSE, Molecular Probes). Varying numbers of DC were co-incubated with 2.5 x 10^4 CFSE-labeled T cells at 37°C/5% CO₂ for 66 hr. After co-incubation, cells were stained with PerCP-Cy5.5-conjugated anti-CD8α and allophycocyanin-conjugated anti-Vα2 TCR (B20.1; eBioscience), as described above. T cell proliferation was assessed by dilution of the CFSE signal. The efficiency of DC enrichment was determined by staining enriched DC with PE-Cy5-conjugated anti-CD11c (N418, eBioscience) and performing flow cytometry as described above.
14. Statistical analyses

Comparisons were made using an unpaired Student’s T-test. When analyzing titers of infectious virus, data were log$_{10}$ transformed prior to analysis. Statistical significance was indicated by $p < 0.05$. 
C. Results

1. HSV pathology is exacerbated by stress applied early in infection

Many studies that have examined immune responses of infected hosts undergoing stress used models in which stress was applied for extended periods of time before, during, and throughout the entire course of an infection and the resulting immune response. In our studies we wanted to examine 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 relatively resistant to HSV infection as compared to other mouse strains [312], were subjected to one session of restraint stress prior to bilateral footpad infection with HSV-1. To limit stress to the early stages of infection, mice were subjected to only three additional daily sessions of restraint stress post-infection. The footpads of stressed or non-stressed mice were photographed daily for 11 days beginning on the first day following infection. Images were scored blindly to assess the severity of HSV lesions over the course of infection. In non-stressed controls, lesions appeared on day 4 post-infection, were most severe on days 5 and 6, and began to resolve beginning on day 7. In stressed mice, the viral lesions were detectable starting on day 3 post-infection and did not begin to resolve until day 8. Thus, the onset of HSV lesions was accelerated and their resolution delayed when stress was applied at the early stages of infection (Fig. 12).

We measured the viral load in the footpads of stressed and control mice at various times post-infection. The footpads of stressed mice contained significantly higher (3-fold) viral titers on the peak of infection (day 5) than the footpads of non-stressed mice,
Figure 12. Restraint stress delays the resolution of HSV lesions on footpads. Digital photographs of footpads from control and stressed HSV-infected mice were blind-coded, scrambled, and scored for the severity of lesions. Data shown are mean ± SE; n = 15 mice/group. Asterisks (*) represent significant difference between stress and control (p < 0.05).
Figure 12

Lesion Score vs. Days

- Control
- Stress

Significance indicated by *
and infectious virus persisted in the footpads of stressed mice for an additional 2 days compared to control mice (Fig. 13). Because HSV has not been detectable in draining LN by plaque assay [313], viral DNA in the draining PLN was measured by quantitative Real Time PCR at daily time intervals post-infection, revealing a peak at 3 days post-infection (Fig. 14A). On day 3 post-infection, there was a 4-fold increase in the amount of HSV DNA in the PLN of the stressed animals compared to non-stressed controls (Fig. 14B).

2. Stress terminated early after infection is sufficient to impair the HSV-specific CD8⁺ T response in the PLN

CD8⁺ T cells provide important protection during HSV infection [174]. Previous studies have demonstrated impaired CD8⁺ T cell responses against viral pathogens when the animals experienced stress throughout the T cell expansion and contraction phases [232]. Following bilateral cutaneous footpad infection with HSV-1, we examined the draining PLN for antigen-specific CD8⁺ T cell responses that were generated after stress was terminated. As above, stress was administered one day prior to infection and stopped 3 days post-infection. On day 5 post-infection, the peak of T cell activation [314], the PLN were removed, and the CD8⁺ T cell response was measured. The number of activated CD8⁺ T cells was measured by intracellular staining for granzyme B. HSV gB<sub>498-505</sub>-specific CD8⁺ T cells were identified by tetramer staining, and the functional properties of these cells were determined by measuring antigen-specific degranulation and the production of IFNγ. In stressed animals, the total numbers of CD8⁺ T cells producing granzyme B were reduced by 3.9-fold, and HSV-1 gB<sub>498-505</sub>-specific CD8⁺ T
Figure 13. Stressed mice have higher HSV-1 load. Footpads were taken from stressed or control mice at various times post-infection, homogenized and infectious virus was quantified by plaque assay. Data shown are mean ± SE; n = 3 for days 1, 3, and 9. Data for days 5 and 7 were compiled from 2 independent experiments; n = 8-9 mice/group. Asterisks (*) represent significant difference from control ($p < 0.05$).
Figure 13

![Graph showing the comparison of PFU (×10^-6)/footpad between Control and Stress groups over days post infection. The graph indicates a significant increase in PFU in the Stress group on days 5 and 7, marked with asterisks.]
**Figure 14.** Stressed mice have more HSV-1 DNA in their PLN. (A) Timecourse of HSV DNA in PLN. DNA was isolated from PLN of stressed or control mice at various days post-infection. Quantitative real-time PCR was performed to measure HSV genomes. n = 2-3 mice for each timepoint. (B) HSV DNA in PLN on day 3 post-infection. Data shown are mean ± SE, compiled from two independent experiments; n = 8 mice/group. Asterisk (*) represents significant difference from control (p < 0.05).
Figure 14

A

- Control
- Stress

Time (dpi)

B

HSV DNA (PFU equivalents/LN)

Control Stress

*
cells were reduced by 2.4-fold as compared to non-stressed controls (Fig. 15A, B). Functional measures of HSV-1 gB\(_{498-505}\) specific CD8\(^+\) T cells in stressed animals showed a 2.9-fold reduction of cells that were able to degranulate and a 5.0-fold reduction of cells producing IFN\(\gamma\) in response to specific peptide (Fig. 15C, D). Thus, even after stress had been terminated, the PLN of stressed mice contained fewer activated CD8\(^+\) T cells and HSV gB\(_{498-505}\)-specific cells at the time of peak T cell activation, and the function of the antigen-specific cells was impaired.

3. Stress impairs antiviral CD8\(^+\) T cell responses via the GR

The previously reported stress-induced failures of both natural and immunization-induced antiviral immunity were mediated primarily by the GC, CORT [9, 12, 283]. In our studies, mice were administered either VEH alone or the GR antagonist RU-486, which blocks CORT binding. GR antagonist was administered daily, 2 hr prior to stress, and mice were subjected to stress and HSV infection described above (restraint stress administered one day prior to footpad infection and terminated days 3 post infection). After 5 days post-infection, PLN were removed to measure CD8\(^+\) T cell responses as described above. The T cell responses in stressed mice that did not receive the GR antagonist were reduced similar to the responses shown in Fig. 15. However, the administration of GR antagonist completely prevented stress from reducing the numbers of activated CD8\(^+\) T cells and HSV-1 gB\(_{498-505}\)-specific CD8\(^+\) T cells (Fig. 16A, B), and the numbers of HSV-1 gB\(_{498-505}\)-specific CD8\(^+\) T cells that were able to undergo degranulation (Fig. 16C), or synthesize IFN\(\gamma\) in response to specific peptide (Fig. 16D).
Figure 15. Stress impairs the CD8+ T cell response against HSV. Stress reduced (A) total number of granzyme B-expressing CD8+ T cells, (B) the number of gB498-505-specific CD8+ T cells, and numbers of functional antigen-specific CD8+ T cells as assessed by (C) degranulation, and (D) IFNγ production. Cells were obtained from the PLN on day 5 post-infection. Data shown are mean ± SE; n = 5 mice/group. Asterisks (*) represent significant differences from control (p < 0.05).
Figure 15
Figure 16. The effects of stress on the CD8$^+$ T cell response are mediated through the GR. The administration of GR antagonist (RU-486) prevented stress from reducing the number of (A) granzyme B-producing CD8$^+$ T cells and antigen-specific CD8$^+$ T cells as assessed by (B) tetramer staining, (C) peptide-specific degranulation, and (D) peptide-specific IFN$\gamma$ production in PLN on day 5 post-infection. Data shown are mean ± SE, compiled from 2 independent experiments; n = 4-7/group. Asterisks (*) represent significant differences from control ($p < 0.05$).
Figure 16
4. Stress-induced impairment of CD8$^+$ T cell responses is not due to direct effects of CORT on the T cells

Previous studies have implicated T cells as the direct targets of stress-mediated anti-viral immunosuppression. However, because GR is expressed by all nucleated cells, no distinction has been made \textit{in vivo} between the action of stress and/or CORT on T cells vs. cells required for their activation, particularly in the context of an antigen-specific infection. To address this issue, we used a knockout mouse strain whose T cells are genetically deficient for the GR (GR-T$_{KO}$) [309]. The lack of GR expression in Thy-1$^+$ thymocytes from Cre$^+$ mice was confirmed by Western blots for GR. Cre$^-$ littermates, which are phenotypically identical to wild-type C57BL/6 mice, were used as controls. We detected GR in CD11c-enriched splenic DC from Cre$^+$ mice and Cre$^-$ littermate controls and found equivalent expression of GR in both groups of mice (Fig. 17A). Whole thymic tissue from each mouse was screened by Western blot to confirm the expression of GR in Cre$^-$ mice and the absence of GR by T cells in GR-T$_{KO}$ mice for all subsequent experiments described here.

GR-T$_{KO}$ mice or Cre$^-$ littermates received drinking water supplemented with VEH or CORT at a dose that results in serum concentrations of CORT \textit{in vivo} that are comparable to those measured in stressed animals [238, 315]. Similar to our restraint stress paradigm, mice received CORT or VEH for 1 day prior to and 3 days following HSV infection, and then returned to normal drinking water. At 5 days post-infection (2 days after termination of CORT treatment), PLN were removed to measure gB$_{498-505}$-specific and functional CD8$^+$ T cells, as described for Figs. 14 and 15. Fig. 17B shows that in Cre$^-$ mice that received CORT, the numbers of HSV-1 gB$_{498-505}$-specific CD8$^+$ T
Figure 17. CORT 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 DC from Cre$^+$ and Cre$^-$ mice. (B) CD8$^+$ T cell responses in CORT or VEH-treated mice were assessed by collecting CD8$^+$ T cells from PLN on d 5 post-infection and staining with HSV-gB-specific tetramer and measuring granzyme B expression and peptide-specific degranulation and IFN$\gamma$ synthesis. Data shown are mean ± SE; n = 2-7/group.
Figure 17

A

<table>
<thead>
<tr>
<th>Cre+</th>
<th>Cre-</th>
<th>Cre+</th>
<th>Cre-</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>T cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

- **Granzyme B+ CD8 T cells (x10^4)**
  - Veh
  - Cort

- **HSV-gB CD8 T cells (x10^4)**
  - Veh
  - Cort

- **CD107+ CD8 T cells (x10^4)**
  - Veh
  - Cort

- **IFN-γ CD8 T cells (x10^4)**
  - Veh
  - Cort
cells were significantly reduced, and functional CD8\(^+\) T cells were suppressed as assessed by granzyme B synthesis, IFN\(\gamma\) production, and degranulation. Similarly, CD8\(^+\) T cell responses were also suppressed in Cre\(^+\) GR-T\(_{KO}\) littermates that received CORT. To determine the effects of stress in these mice, Cre\(^+\) GR-T\(_{KO}\) mice or Cre\(^-\) littermates were subjected to restraint stress for 1 day prior and 3 days following HSV infection. At 5 days post-infection, PLN were removed and HSV-specific CD8\(^+\) T cell function was assessed as above. Similar to the results seen with CORT, restraint stress had a significant suppressive effect on the generation of antigen-specific and functional CD8\(^+\) T cells in Cre\(^-\) mice, while in the GR-T\(_{KO}\) mice CD8\(^+\) T cell responses were not restored (Fig. 18A). 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 CORT on the T cells.

5. Impaired control of viral replication is not due to direct effects of stress-induced CORT on T cells

We had determined that stress-induced CORT at the time of infection prolonged the time course of lesions in footpads (Fig. 12) and increased HSV load in footpads and PLN (Fig. 13). We had also found that stress and CORT reduced HSV-specific CD8\(^+\) T cell numbers and function via a mechanism in which CORT did not affect the T cells directly (Figs. 17, 18A). We therefore asked whether direct effects of stress-induced CORT on T cells were responsible for the impaired control of viral replication. We measured the amount of infectious virus in the footpads from the GR-T\(_{KO}\) Cre\(^+\) and Cre\(^-\) mice used in the previously described experiment. Stressed Cre\(^-\) mice had a 3.7-fold increase in viral PFU/footpad compared with non-stressed Cre\(^-\) mice (Fig. 18B). Cre\(^+\)
**Figure 18.** Stress suppresses CD8$^+$ T cell responses and limits control of viral replication independently of direct effects on T cells. (A) CTL responses in control or stressed mice were assessed by collecting CD8$^+$ T cells from PLN on day 5 post-infection and staining with HSV-gB$_{498-505}$-specific tetramer and measuring granzyme B expression and peptide-specific degranulation and IFN$\gamma$ synthesis. (B) Footpads were removed from stressed or control mice on day 5 post-infection, homogenized and infectious virus was quantified by plaque assay. Data shown are mean ± SE; n = 5-8/group.
Figure 18
GR-TKO littermates that were stressed also had significantly more virus in their footpads, a 6.9-fold increase compared with non-stressed Cre+ GR-TKO mice (Fig. 18B). These data show that the presence or absence of the GR on T cells had no impact on the amount of infectious virus in the footpads of stressed mice, therefore impaired control of viral infection was not due to direct effects of stress-induced CORT on T cells.

**6. DC function is impaired by stress in vivo**

DC are highly specialized for the acquisition, processing, and presentation of antigen, and are the most efficient of all pAPC [316]. Previous studies in our laboratory have demonstrated that CORT impairs DC maturation and antigen presentation, such that CORT-treated DC are less able to prime naive or antigen-specific CD8+ T cells [13] (Chapter III). In the present 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 CORT on T cells. We therefore asked whether stress impairs the function of DC from HSV-infected mice, as DC are critical for CD8+ T cell activation. For these experiments, mice were subjected to stress for one day prior to and one day following footpad HSV infection. DC were enriched from the PLN of stressed or non-stressed mice 24 hr after infection, and equivalent numbers of enriched DC were co-cultured with CFSE-labeled, HSV-specific CD8+ T cells from TCR transgenic HSV gB-T-1.3 mice. Because the efficiency of purification can vary, we determined the percentage of DC in each preparation to ensure that the same numbers of DC were present in each co-culture. Proliferation was assessed by the dilution of CFSE signal. As was expected, naive DC alone or DC pulsed with an irrelevant peptide (OVA257-264) did
not induce proliferation of CD8^{+} HSV-1 gB-T1.3 cells (Fig. 19A). However DC from the PLN of stressed, HSV-infected mice were substantially less efficient at inducing proliferation compared to DC from non-stressed mice (Fig. 19B, C), demonstrating that stress had impaired the ability of these DC to prime antigen-specific CD8^{+} T cells. These data provide strong evidence that the observed suppression of CD8^{+} T cell responses in animals undergoing psychological stress is due to impairments in DC function.
Figure 19. Stress impairs the ability of DC from HSV-infected mice to induce proliferation by antigen-specific CD8+ T cells. DC were purified from the PLN of HSV-infected stressed or control mice 24 hr post-infection. 1.3 x 10^4 DC were co-cultured with 2.5 x 10^4 CFSE-labeled CD8+ cells from the spleen of a naive gB-T transgenic mouse, specific for HSV gB_{498-505}. (A) DC from a naive mouse, pulsed with an irrelevant peptide (OVA_{257-264}), did not induce proliferation. (B) DC from non-stressed mice were able to induce T cell proliferation. (C) Equivalent numbers of DC from stressed mice were less efficient at stimulating proliferation compared to non-stressed controls. Data shown are representative of three independent experiments.
D. Discussion

Many studies examining the effects of psychological stress on adaptive immunity employed prolonged exposure to stress, beginning up to 4 days prior to infection and continuing for as long as 8 days post infection [6, 12, 14, 264, 283]. In the present study, exposure to stress was limited to 24 hr prior to infection and continued for 1 to 3 days following infection. This protocol allowed us to determine the impact of stress on the earliest stages of the generation of an immune response and how such interference affected the subsequent outcome of an infection. While it has been firmly established that chronic psychological stress substantially impairs CD8$^+$ T cell responses, it has been difficult to distinguish the extent to which this impairment is due to direct effects of stress-induced neuroendocrine factors on T cells themselves vs. effects on DC required for their activation. Here we accomplished this distinction using GR-T$^+$KO mice, whose T cells do not express GR. A number of studies have identified effects of GC on various properties of DC, typically \textit{in vitro} [13, 272] (Chapter III) and/or using pharmacological doses of synthetic GC [271, 274, 295]. The work presented here uses a variety of approaches within \textit{in vivo} systems to delineate the events and mechanisms underlying of the effects of a physiological stressor on DC function during a viral infection.

From our results, we can construct a chronology of the effects of stress on the immune response to a footpad HSV infection. DC have been shown to present antigen within 6 hr following footpad infection by vaccinia [284] and HSV [317]. Mice depleted of DC prior to footpad HSV infection succumbed with 100% mortality in 3-6 days following infection [168]. In our study, utilizing one session of stress prior to HSV infection and one session post-infection, we found that within 24 hr post-infection DC
were already substantially impaired in their ability to prime HSV gB<sub>495-505</sub>-specific CD8<sup>+</sup> T cells (Fig. 19). Furthermore, after stress has been terminated, we found that the quality and size of the subsequent CD8<sup>+</sup> T cell response was reduced (Fig. 15). It is established that without a CD8<sup>+</sup> T cell response, HSV clearance is delayed [180], similar to what we found in mice that had been stressed at early times post-infection (Fig. 13). Thus, stress interferes with the earliest stages of DC function, which has substantial consequences on the magnitude and efficacy of the subsequent T cell response to HSV, leading to delayed viral clearance and more severe viral pathogenesis.

Our previous studies have demonstrated that physiological concentrations of the GC CORT impair DC function, rendering DC less efficient for CD8<sup>+</sup> T cell priming in vivo (Chapter III). Here we show that CORT was responsible for mediating the effects of stress on the CD8<sup>+</sup> T cell response to an HSV infection (Fig. 16). Although other immunomodulatory hormones are produced during stress, such as catecholamines [206] and endogenous opioids [318], we found that stress-induced suppression of the CD8<sup>+</sup> T cell response was blocked by the administration of GR antagonist. By using the GR-T<sub>KO</sub> mice we found that stress or exogenous CORT significantly impaired CD8<sup>+</sup> T cell responses and control of viral load despite a lack of GR expression by the T cells themselves (Figs. 17, 18). Since DC in GR-T<sub>KO</sub> mice expressed normal amounts of GR (Fig. 17A), these cells remained susceptible to CORT.

DC are a heterogeneous population of cells that participate in a complex array of both innate and adaptive immune functions [291]. LN-resident CD8α<sup>+</sup> DC are thought to be important for cross-presentation of viral antigen [141] and contribute to antigen presentation during dermal HSV infections [165]. pDC, which secrete large amounts of
type I IFN in response to viral infections, are critical for innate immunity during mucosal HSV infection [49] and may promote the priming of CD8\(^+\) T cells by cDC in cutaneous HSV infections [61]. Recent work by our lab shows that both the CD8α\(^+\) DC and pDC subsets are preferentially highly susceptible to the effects of stress-induced CORT, and that cross-priming of antigen-specific CD8\(^+\) T cells is significantly impaired in stressed mice in a GR-dependent manner (Hunzeker and Truckenmiller, unpublished observations).

Our earlier studies provide insight into the cellular and molecular mechanisms by which stress-induced GC suppress DC function [287]. We have demonstrated that CORT renders DC less efficient for priming antigen-specific CD8\(^+\) T cells in vivo (Chapter III) and impairs antigen presentation by virally-infected DC [13]. Impaired antigen presentation by DC 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 ER [13]. We have also shown that CORT impedes the ability of DC to produce pro-inflammatory cytokines (IL-6, IL-12, and TNFα) in response to TLR stimulation (Chapter III). The present study demonstrates that impaired DC function has deleterious consequences for the stressed host in the context of an HSV infection.

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. When animals were subjected to stress prior to infection, their DC function was quickly impaired, leading to the inefficient generation of a CD8\(^+\) T cell response. Together these effects resulted in higher viral loads with earlier
onset and delayed resolution of skin lesions several days after stress was stopped (Fig. 12). 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. The temporary state of 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.
CHAPTER V. ADDITIONAL FINDINGS

In addition to the studies on the effects of CORT on DC function \textit{in vitro} (presented in Chapter III) and the studies on the role of stress-induced suppression of DC function \textit{in vivo} during HSV infection (presented in Chapter IV), several additional experiments have been performed to further characterize the effects of CORT on DC function. While the results of these experiments have not been published in the form of a complete manuscript, their findings have relevance to the overall understanding of how stress and stress-induced GC affect DC function and viral pathogenesis, both \textit{in vitro} and \textit{in vivo}.

A. Stress does not impair DC maturation in PLN during HSV infection \textit{in vivo}

Since our findings presented in Chapter III established that physiological “stress levels” of CORT (1 µM) were able to impair maturation of BMDC in response to TLR ligation \textit{in vitro}, we wanted to determine whether stress could impair DC maturation \textit{in vivo}. To address this question, we measured the surface expression of the costimulatory molecule B7.2 by DC in the PLN following footpad HSV infection.

Mice were subjected to one session of restraint stress prior to bilateral hind footpad HSV or mock-infection. HSV (3 x 10^5 PFU) or diluent (PBS 1% FBS) was administered in a volume of 4 µl via the multiple piercing method. Mice were subjected to one additional session of restraint stress, and PLN were collected. LN tissue was digested using Collagenase D and homogenized into a single cell suspension. Cells were then stained for Thy-1.2, CD19, NK1.1, CD11c, and B7.2 and expression was measured by flow cytometry. DC were identified as Thy-1.2^− CD19^− NK1.1^− CD11c^+ cells, and the
percentage of DC expressing high levels of B7.2 was determined by analysis using FlowJo.

Although CORT treatment was able to suppress LPS-induced DC maturation \textit{in vitro} (Fig. 2), a large percentage of DC in the PLN of stressed HSV-infected mice expressed high levels of B7.2, similar to what was seen in non-stressed mice (Fig. 20). These findings indicate that DC in the PLN of both groups of mice had matured in response to HSV infection, as the PLN of mock-infected mice from each group contained a significantly lower proportion of DC that were B7.2^{HI}. 
**Fig. 20.** Stress does not inhibit DC maturation in the PLN of HSV-infected mice. Data are combined from two separate experiments with each group containing 2-3 mice/group and are presented as percentages of DC that expressed high levels of B7.2. * = $p < 0.05$. 
Figure 20

% B7.2^+ (CD11c^+, dump^-)

FWD

RST

Mock

HSV

* *
B. CORT treatment does not impair macropinocytosis by DC

Previous studies have found that CORT suppresses direct presentation of viral antigen on MHC class I [13]. Ongoing studies have found that cross-presentation is also impaired *in vivo* by stress and *in vitro* by CORT (Hunzeker et al., manuscript in preparation). Since DC must take up exogenous antigen in order for cross-presentation to occur, defects in antigen uptake could result in impaired cross-presentation. Data from experiments on the effect of CORT on the uptake of soluble protein have shown that CORT does not inhibit endocytosis, but instead renders DC resistant to LPS-induced downregulation of endocytosis (Fig. 7). In addition to endocytosis, DC use macropinocytosis or fluid phase uptake to acquire and concentrate antigen [319]. To determine the effect of CORT on macropinocytosis, we measured the rate of uptake of Lucifer Yellow (LY), a soluble tracer.

BMDC were generated, treated with CORT (1 µM) or VEH (0.1% EtOH) for 48 hr, and enriched using CD11c microbeads as described in Chapter III. CD11c+ cells were incubated in prewarmed (37 °C) complete phenol red-free IMDM (Invitrogen) for 30 min. LY (Molecular Probes) was added at a final concentration of 300 µg/ml. Cells were incubated at 37 °C, and 3 x 10^5 cells were removed at 20 min intervals and transferred to ice-cold PBS 1% FBS. Cells were transferred to black 96-well plates (Dynex Technologies, Chantilly, VA), washed extensively, and lysed in PBS containing 0.3% Triton-X-100. Lysates were analyzed for fluorescence using an XFluor4 Safire II plate reader (Tecan, Research Triangle Park, NC). Samples were excited at a wavelength of 428 nm, and emissions were read at 544 nm. Cells incubated with LY on ice served as negative controls.
We found that CORT-treated BMDC were equally able to take up LY as VEH-treated DC (Fig. 21). This demonstrates that CORT does not impair cross-presentation by downregulating macropinocytosis.
**Figure 21.** CORT has no effect on macropinocytosis by BMDC. CORT (open squares) or VEH (closed circles)-treated DC were incubated with LY at 37°C (solid line) or on ice (dashed line). Aliquots were collected at 20’ intervals, washed extensively, and lysed. LY uptake was measured by fluorimetry. Data are shown for the average of three separate experiments and are expressed in relative fluorescence units.
Figure 21

- Vehicle
- Cort
C. CD8α⁺ DC and pDC in the PLN are preferentially susceptible to stress-induced CORT

Associated with stress-induced impairment of DC function is a decrease in the number of DC in secondary lymphoid tissues. Ongoing studies have examined the effects of stress and stress-induced GC on various DC subsets in these tissues and compared how stress affects the number of CD8α⁺ DC, CD11b⁺ DC, and pDC, which are B220⁺ CD11b⁻. In these studies, mice were subjected to four sessions of restraint stress and DC subsets in the spleen were identified and counted. These experiments show that CD8α⁺ DC and pDC in the spleen are preferentially more susceptible to stress, as the frequency of these cells amongst other splenocytes is reduced to a greater extent than other cells, including CD11b⁺ DC (Truckenmiller et al., unpublished observation). However, it was not known whether the CD8α⁺ DC and pDC in draining LN were also more susceptible to stress. To address this question, the numbers of CD8α⁺ DC, CD11b DC, and pDC in PLN were determined in stressed and non-stressed mice by flow cytometry.

Since these studies were performed in conjunction with the studies on DC maturation (presented in Fig. 20), mice were subjected to one session of restraint stress before mock-infection of both hind footpads with 4 µl PBS 1% FBS via the multiple piercing method. Mice were then subjected to an additional session of restraint stress following mock-infection. PLN were collected 24 hr post mock-infection and were digested using Collagenase D to obtain a single cell suspension. Total PLN cells were counted and stained for Thy-1.2, CD19, NK1.1, CD8α, CD11b, B220, and CD11c. DC subsets were identified using flow cytometric analysis.
The results showed that stressed mice had significantly fewer CD8α⁺ DC and pDC in the PLN compared to non-stressed controls (Fig. 22). In contrast, the number of CD11b⁺ DC in the PLN of stressed mice remained unchanged, compared to controls, suggesting that these cells may be resistant to the effects of CORT.
Figure 22. CD8α^+ and pDC in the PLN are preferentially susceptible to stress-induced depletion. Numbers of each subset of DC were normalized between two independent experiments, each containing data from 3 mice/group. * = p < 0.05.
Figure 22

% Cell Number (rel. Control)

CD8 | CD11b | Plasmacytoid

Control | Stress

*
D. Naltrexone does not restore splenic cellularity in C57BL/6 and Balb/c mice

While several studies described in this dissertation are concerned with the effects of stress-induced GC on immune function, other studies have suggested that stress-induced opioids are important for mediating a portion of the effects of stress on immune cells. One study demonstrated that stress-induced apoptosis of splenocytes was mediated by endogenously-produced opioids by using the opioid receptor antagonist naltrexone to prevent decreased splenic cellularity in stressed mice [318]. The role of opioids in immune cell apoptosis were confirmed using mice that were deficient for the µ-opioid receptor [320]. With the intent to determine the role of opioids in suppressing immunity against HSV, a pilot study was performed to replicate these findings.

C57BL/6 or Balb/c mice were injected with naltrexone (200 µg) or PBS i.p. in a volume of 200 µl 1 hr prior to each stress session. Mice were then subjected to two 16 hr sessions of restraint stress. Immediately following the second session of stress, spleens were obtained and digested with collagenase D into a single cell suspension. Splenocytes were then counted to determine the total cellularity of the spleen. Since previously reported studies used Balb/c mice or Balb/c X C57Bl/6 F1 mice, we included both C57Bl/6 and Balb/c mice in our study.

Although stressed mice showed a trend toward reduced splenic cellularity, we were surprised to find that naltrexone was unable to restore cellularity in either strain tested (Fig. 23). The discrepancy between these findings and those reported in the literature cannot be explained at this time.
**Figure 23.** Naltrexone fails to restore splenic cellularity in stressed mice. Data are from experiments using (a) C57Bl/6 and (b) Balb/c mice. Each experiment included 2-3 mice per group. * = p < 0.05.
Figure 23

(a)  

Cells x 10^{-7} / Spleen  

Control  Stress

(b)  

Cells x 10^{-7} / Spleen  

PBS  Naltrexone
E. HSV-infected mice subjected to stress in the early stages of infection have less viral DNA in dorsal root ganglia

Although we have shown that stress administered during the earliest stage of HSV infection compromises the subsequent generation of an antiviral CD8$^+$ T cell response and results in increased viral load and delayed clearance, it was not known whether this exposure to stress affected the ability of HSV to establish latency in neuronal tissue. Previous studies have demonstrated that HSV-specific CD8$^+$ T cells reduce the ability of HSV to establish latency in sensory ganglia [174] and that stress-induced depletion of CD8$^+$ T cells permits increased viral reactivation [268]. If exposure to stress in the earliest stages of an HSV infection was able to suppress the subsequent immune response, then it was thought likely that this suppression could facilitate the establishment of latency by HSV in the dorsal root ganglia (DRG) that enervate the footpad.

To test this hypothesis mice were subjected to restraint stress for 1 day prior to and three days following bilateral HSV-1 footpad infection. One year after infection mice were exanguinated by cardiac puncture and the L$_4$, L$_5$, and L$_6$ DRG were collected. Total DNA was isolated and quantitative real-time PCR was performed to measure HSV genomic DNA as described above.

HSV genomic DNA was detected in tissue from latently infected non-stressed mice. Unexpectedly, we found 3-fold less viral DNA in tissues from stressed mice (Fig. 24). Although this experiment has not been repeated, it was unexpected that DRG from stressed animals should contain fewer copies of viral DNA, since the immune response against HSV was suppressed following infection.
Figure 24. Exposure to stress early in infection reduces the amount of HSV DNA in the DRG. Genomic HSV DNA was measured in DRG from latently infected mice by quantitative real-time PCR. Data presented are from a single experiment with 12-14 mice per group. * = p < 0.05.
Figure 24

Viral DNA (pFU equiv. per mouse)

FWD

RST

0 20 40 60 80 100

*
CHAPTER VI. DISCUSSION

While it is understood that stress-induced GC can suppress immunity to infection, the cellular targets of this GC and the cellular and subcellular mechanisms by which immune function is impaired remain unknown. The studies presented in this dissertation have identified one such target as DC and suggested that stress-induced suppression of DC function contributes to the failure of the mammalian immune system to successfully initiate CD8$^+$ T cell responses and control viral infection. In Chapter III, we have determined that the stress-associated GC CORT impairs the ability of DC to respond to LPS and other TLR stimuli. CORT impaired the surface expression of various maturation markers, blocked LPS-induced downregulation of endocytosis, and attenuated the production of pro-inflammatory cytokines, altogether resulting in a decreased ability of adoptively transferred DC to prime naive CD8$^+$ T cells \textit{in vivo}. In Chapter IV, we extended our studies \textit{in vivo} to determine how stress might affect the earliest stages of an antiviral immune response and what consequences, if any, these effects have on the course of infection. From these studies, we made several independently significant findings:

1. Early exposure to stress increases viral load at the site of infection and delays both viral clearance and resolution of viral lesions, several days after stress is terminated.

2. Stress suppresses CD8$^+$ T cell responses in a GR-dependent manner by the direct action of stress-induced CORT on non-T cells.

3. Stress impairs the ability of DC in the draining PLN of HSV-infected mice to induce proliferation by naive, antigen-specific CD8$^+$ T cells.
Together these findings suggest that one mechanism by which stress-induced CORT impairs immunity to HSV is via the suppression of DC function, which leads to impaired CD8$^+$ T cell priming. A weaker CD8$^+$ T cell response likely fails to clear infectious virus efficiently, resulting in exacerbated disease and pathology. Our findings in Chapter III provide strong evidence to suggest that numerous aspects of DC function are modulated by CORT.

A. Implications for a model of CORT-induced impairment of DC function

The experiments presented in this dissertation provide evidence that stress-induced CORT suppresses DC function, and that this suppression is responsible for impaired T cell responses and failure to control viral infection. Despite this evidence, this model of stress-induced immunosuppression relies on several assumptions and raises certain questions:

1. Although CORT-treatment could clearly impair DC maturation \textit{in vitro}, DC isolated from the PLN of HSV-infected mice that had been subjected to stress were predominantly mature (B7.2$^{HI}$, Fig. 20). What might account for this discrepancy?

2. Experiments in Chapter IV demonstrate the DC function is impaired in the PLN of stressed mice and that stress-induced CORT suppresses CD8$^+$ T cell responses via direct effects on non-T cells. It is assumed that this observation is due to the effects of CORT on DC. Are DC truly the direct cellular target of CORT?
3. Experiments in Chapter IV also demonstrate that the impaired CD8$^+$ T cell response in stressed mice is associated with increased viral load at the site of infection, and delayed resolution of viral lesions. Is the impaired T cell response in stressed animals responsible for these effects on the course of infection?

The answers to these questions cannot be determined from the results presented in this dissertation but remain to be addressed by future studies. It is possible, however, to speculate on the concepts and strategies that could be employed to extend the findings presented here.

1. **What accounts for the discrepancy between *in vitro* effects of CORT and *in vivo* effects of stress on DC maturation?**

Several studies in diverse areas of biology have revealed phenomena that are discovered in tissue culture models only to be refuted by further exploration *in vivo*. It must never be assumed that tissue culture can truly model the complex biological, chemical, and physical intricacies at work in a living organism. In our studies, there are several possible reasons that may account for the discrepancy between our findings *in vitro* and *in vivo*.

a. **CORT does not equal stress**

In our *in vitro* studies presented in Chapter III, we treated DC with 1 µM CORT, a concentration that has been measured *in vivo* in stressed animals [230]. However, in stressed animals, true concentrations of CORT vary with the circadian rhythm, resulting
in a time-dependent variation in CORT concentrations that is difficult to recapitulate in vitro [237]. The bioavailability of CORT is regulated by CBG, which can sequester extracellular CORT and 11β-hydroxysteroid dehydrogenases, the enzymes responsible for the interconversion of GC between active and inactive forms [211]. These concentration and activity of these proteins can have substantial effects on local concentrations of bioavailable CORT. Furthermore, several other non-GC hormones are produced during stress including endorphins, catecholamines and MSH, which are not reflected in our in vitro model. It is possible that one or more of these hormones may counteract the suppressive effects of CORT on DC maturation.

b. LPS does not equal HSV

It is readily apparent that immersing cells in a solution of LPS is not a true physiological representation of an in vivo phenomenon, but is especially relevant when drawing comparisons to DC that activated during HSV-infection. LPS signals through surface-expressed TLR4 [19], whereas HSV is known to trigger cells through TLR2 [51] and TLR9 [48]. In addition to TLR signaling, which may be suppressed by CORT, HSV may also induce DC maturation via interactions with cytosolic DNA sensors [50]. The exact mechanisms by which HSV induces DC maturation remain unclear. However, it is unlikely that DC are productively infected by HSV virions to induce maturation, as in vitro studies have found that direct infection of DC with HSV prevents DC from maturing [321].
c. BMDC are not equivalent to DC found in vivo

The method used in Chapter III to generate BMDC in vitro from bone marrow-derived precursors results in the differentiation of immature CD11b\(^+\) DC. Although these cultures were not completely homogeneous, they consisted of predominantly one subset of CD11b\(^+\) DC. These cells do not adequately represent the wide range of DC subsets found in vivo, which include CD8\(^+\) DC, CD4\(^+\) DC, and pDC [291]. DC are a relatively rare cell type in any given tissue. In vivo, DC are surrounded by parenchymal tissues in the periphery or T cells in the T cell zones of draining LN. Interactions with these other cells, which do not occur in vitro, are likely to play an important role in regulating DC maturation [122].

d. In vivo studies may not have been able to detect impairment of DC maturation

According to the traditional dogma of DC biology, immature DC are found in peripheral tissues and undergo maturation and migration to LN after encountering a maturation-inducing stimulus. It may be possible that stress was able to impair DC maturation but immature DC were not detected, as they failed to migrate to the draining PLN. One of the changes that maturing DC undergo is the upregulation of the chemokine receptor CCR7 [119]. CCR7 promotes cellular migration to secondary lymphoid tissues, such as LN. The effects of stress-associated hormones on the expression and signaling of CCR7 or the production and secretion of its ligands, macrophage inflammatory protein (MIP)-3\(\beta\) [322] and secondary lymphoid chemokine (SLC) [323] are unknown. Studies that track the fate of DC in peripheral tissues could determine whether stress causes
immature DC to remain in peripheral tissues, even in the presence of stimuli that might otherwise induce DC maturation.

2. What are the cellular targets for stress-induced CORT?

In Chapter IV, we found that stress or CORT impaired the CD8$^+$ T cell response without the need for stress-induced CORT to act directly on T cells themselves. The suppressive effect of stress was blocked by administration of a GR antagonist, demonstrating that stress-induced CORT was responsible for suppressing CD8$^+$ T cell function. Since CORT is required to suppress CD8$^+$ T cell responses, but does not need to act directly on CD8$^+$ T cells themselves, then we can deduce that CORT impairs the function of another cell type that is required for efficient CD8$^+$ T cell responses.

In our GR-T$_{KO}$ mice, CD4$^+$ T cells are deficient for GR as well as CD8$^+$ T cells, indicating that potential effects of CORT on T cell help are not required to suppress CD8$^+$ T cell responses. DC in GR-T$_{KO}$ mice express GR, and DC function is impaired by stress. Since antigen presentation by DC is critical for the efficient priming of CD8$^+$ T cells, it is very likely that direct effects of stress-induced CORT on DC may be responsible for a substantial component of the effects of stress on CD8$^+$ T cell responses. If this hypothesis is correct, then the administration of a GR antagonist should restore the ability of DC to present antigen ex vivo, in addition to the restoration of the subsequent CD8$^+$ T cell response. Another way to determine the role of the direct effects of CORT on DC would be to employ CD11c-Cre, GR$^{LoxP/LoxP}$ mice (expressing Cre under the CD11c promoter). The DC in these mice would lack GR in DC, and these mice could be used to determine the effect of GC on DC function. By crossing these mice with GR-T$_{KO}$
mice, the relative contributions of the effects of CORT on T cells and DC could be determined.

It is possible that the cellular targets of CORT that are required for suppressing CD8\(^+\) T cell responses may not be limited to T cells and DC. Other hematopoietic cells, such as monocytes or macrophages, might be susceptible to CORT and fail to produce the necessary cytokines or other factors required for efficient T cell priming. The collective role of hematopoietic tissues in mediating the effects of CORT on suppression of CD8\(^+\) T cell responses, could be examined by using a vector, such as a retrovirus, to transduce Cre into bone marrow of GR\(^{\text{LoxP/LoxP}}\) mice for the construction of bone marrow chimeras [324].

Alternatively, endothelial cells may also be targets of CORT and could impair the efficient extravasation of immune cells into secondary lymphoid tissues. The importance of these tissues could be addressed by crossing GR\(^{\text{LoxP/LoxP}}\) onto a Tie2-Cre or Flk1-Cre background, which would generate mice that are deficient for GR in endothelial tissues [325, 326].

The potential utility in using a series of tissue-specific GR-knockouts could theoretically allow for the extensive characterization of specific cellular targets of CORT. However, it is important to consider that the increased inactivation of GR in various tissues could result in abnormal development or impaired GC homeostasis.

Although we assume that CORT suppresses the function of a cell that is required for CD8\(^+\) T cell activation, it is formally possible that CORT could induce an otherwise inactive cell to actively suppress the generation of T cell responses. Although it may be tempting to speculate that CD4\(^+\) CD25\(^+\) FoxP3\(^+\) T\(_{\text{reg}}\) cells may be induced by CORT, it is
unlikely that such an induction of T\textsubscript{reg} cells is sufficient for the impairment of CD8\textsuperscript{+} T cell responses in our model. Bulk T cells from GR-T\textsubscript{KO} mice were deficient for GR (Fig. 17). While the expression of GR was not examined in specific subsets of T cells (T\textsubscript{reg} cells, NK-T cells, \(\gamma\delta\) T cells), our data suggest that stress-induced impairment of CD8\textsuperscript{+} cell responses occurs independently of the effects of CORT on any type of T cell (CD8\textsuperscript{+}, CD4\textsuperscript{+}, or T\textsubscript{reg}).

It is also possible that several of the immunosuppressive effects attributed to CORT may not be due to direct effects of CORT, but may be due to some other soluble factor or hormone whose production is induced by CORT. Such a hormone could act in a paracrine or endocrine fashion to suppress immune function in other cells. This possibility is thought to be unlikely, as \textit{in vitro} studies often examine the effects of GC on purified or highly enriched populations of cells. One might suggest that these \textit{in vitro}-cultured populations of cells could produce such a factor themselves, but the mere existence of such a factor has yet to be discovered.

While the studies presented in Chapter IV do not explicitly define DC as the direct target of CORT responsible for mediating suppressed CD8\textsuperscript{+} T cell responses, the evidence at hand supports such a theory. In Chapter III, it was firmly established that several aspects of DC function are impaired by CORT, resulting in a decreased ability of DC to prime CD8\textsuperscript{+} T cell responses. In Chapter IV, we found that DC function in the PLN of stressed mice was profoundly impaired. Given the critical role of DC in priming CD8\textsuperscript{+} T cell responses, it is very likely that the direct effects of stress-induced CORT act on DC to contribute to the impaired generation of CD8\textsuperscript{+} T cell responses.
3. Are the increased viral load and delayed viral clearance and resolution of lesions in stress mice due to impaired CD8+ T cell responses?

The studies presented in Chapter IV demonstrate that stress results in impaired CD8+ T cell responses, higher viral load at the site of infection, delayed viral clearance, and delayed resolution of viral lesions. There are several pieces of evidence supporting the hypothesis that the weakened CD8+ T cell response is responsible for impaired control of HSV.

It is well established that CD8+ T cells are important for efficient clearance of HSV. Although CD8+ T cells are not required for protection, depleting these cells results in a prolonged course of HSV infection [180], while the adoptive transfer of activated CD8+ T cells reduces the amount of infectious virus in the footpad and attenuates the establishment of latency in DRG [174].

In our studies, mice were exposed to stress early during the infection, which likely resulted in delayed priming of CD8+ T cells. A delay in CD8+ T cell activation could allow HSV to gain a “foothold” within an organism, allowing for an elevated viral load, resulting in delayed viral clearance. Previous studies on stress-induced HSV encephalitis have found that stressed mice undergoing an intranasal HSV infection had delayed T cell recruitment to the CNS, resulting in increased viral replication and subsequent mortality [5].

The relationship between impaired control of viral replication and a delay in CD8+ T cell responses can be examined experimentally. One method for examining this relationship may involve the use of mice without a functional CD8+ T cell compartment, such as Rag−/− mice. The kinetic role of CD8+ T cells in controlling HSV infection could
be determined by adoptively transferring HSV-specific T cells at various days post infection and measuring the effect of delaying T cell transfer on viral load.

It is also possible that stress could suppress innate immune functions that are required for control of infection. It is known that depleting DC results in 100% mortality in HSV-infected mice within 3-6 days [168]. Since CD8$^{+}$ T cells are not required for survival from HSV, it is likely that DC provide critical innate immune functions that are required to limit viral pathology in addition to their role in T cell priming.
B. Unanswered Questions about the Biology of Stress-induced Immunosuppression

There are several questions that remain to be addressed within the field of neuroimmunology pertaining to stress-induced immunosuppression, but here I will focus on only those most relevant to the studies presented in this dissertation. Two questions stand out as the most appropriate extensions of this work on molecular and cellular scales.

1. What are the mechanisms by which CORT affects DC?

To extend the findings presented in this dissertation on a molecular level, it is necessary to understand the basis for the effects of CORT on DC function on a molecular level. The studies presented in Chapter III provide solid evidence that CORT acts directly on DC to modulate their function. In Chapter IV, compelling evidence was presented that the effects of CORT on DC function have a substantial bearing on the outcome of an antiviral immune response. A better understanding of the molecular actions of CORT within DC will facilitate the design of therapeutic strategies aimed at modulating the effects of CORT to prevent stress-induced immunosuppression.

CORT likely suppresses the immunogenicity of DC by two mechanisms. These mechanisms include effects on DC function and effects on DC viability. While it is clear that CORT is able to modulate DC function by impairing the ability of DC to respond to LPS stimulation, the degree to which various DC functions are impaired varies, depending on which function is studied. VEH-treated DC are able to upregulate B7.1, B7.2, and CD40 in response to LPS stimulation. However, when cells are treated with CORT prior to LPS stimulation, CD40 upregulation appears to be completely resistant to
CORT-mediated suppression, the upregulation of B7.1 is partially upregulated, whereas the upregulation of B7.2 is completely blocked. The molecular mechanisms by which CORT modulates expression of these genes remains unknown. The various means by which GC can modulate gene expression are reviewed in Chapter II. It is possible that different promoters contain GRE with differing affinities for CORT-bound GR or that these elements might lie in more or less critical regions to modulate transcription [327, 328]. Alternatively, CORT-bound GR may interact with (and inhibit) various transcription factors [216], which may be essential for the transcription of some genes but act only to enhance the transcription of other genes. An understanding of the mechanism of action of CORT and GR on the various functions of DC is important to determine the feasibility of therapeutic attempts to restore DC function, and what strategies should be employed in doing so.

In addition to suppressing DC function, it is possible that CORT may reduce the viability of DC. Although our *in vitro* studies did not show any cytotoxic effects of CORT on CD11b⁺ BMDC (Fig. 4), the experiments presented in Chapter V on the various DC subsets suggest that numbers of CD11b⁺ DC in PLN are resistant to the effects of stress. This was not the case for the CD8α⁺ and pDC subsets, which were reduced in number by stress (Fig. 22). It is also possible that these subsets of cells are not killed during stress, but rather are forced to exit or prevented from entering the PLN. In any case, the death or faulty migration of a portion of the DC compartment would result in an overall impairment of DC function, even if remaining cells can function normally. In reality, the question of whether DC are killed or misdirected from the LN is only a part
of a much larger question that remains unsolved in studies on stress-induced immunosuppression.

2. **What is the mechanism that underlies the decrease in cellularity of secondary lymphoid tissues during psychological stress?**

   On an organismal level, one of the most basic observations made in stressed animals is the decreased cellularity of the spleen and LN. Four processes contribute to the cellularity of any organ. These include proliferation, cell death, migration into an organ, and migration out of an organ. Changes in any of these processes result in altered cellularity. In the studies presented in this dissertation, we have observed an impaired CD8$^+$ T cell response to HSV in the PLN of stressed mice. This impairment is detected by the effects of stress on the cellularity of responding CD8$^+$ T cells. By impairing the ability of DC to prime naive CD8$^+$ T cells, CORT reduces the ability of T cells to proliferate. It is also very likely that CORT or other neuroendocrine hormones reduce the size of the pool of HSV-specific CTL precursors by inducing cell death. The effects of CORT and stress on the recruitment and migration of CD8$^+$ T cells are unknown, but it is known that short-term exposure to stress can induce leukocytes to migrate from the blood into cutaneous tissues [200].

   One of the most urgent questions that remains poorly addressed involves the mechanisms underlying changes in the cellularity of secondary lymphoid tissues during stress. While it is important to understand the dynamics responsible for changes in cellularity during infection, the effects of stress on the cellularity of secondary lymphoid tissues during the steady-state are also poorly characterized. An understanding of how
immune responses are affected by stress requires adequate knowledge of the effects of stress on steady-state immune homeostasis, before the additional variables present during an infection can be applied.

While certain attempts have been made to determine the mechanisms by which stress modulates in leukocyte cellularity, further investigations are required. Some studies have reported that endogenously produced opioids are responsible for inducing a decrease in the cellularity of secondary lymphoid tissues [318, 320]. These studies found that apoptosis is induced during stress via the death receptor FAS, which is upregulated by opioids acting partially through the µ-opioid receptor. These studies used opioid receptor antagonists and µ-opioid receptor knockout mice to draw these conclusions. Unfortunately, our attempts to replicate these findings using the opioid receptor antagonist naltrexone failed to find a role for opioids in the stress-induced reduction in splenic cellularity (Fig. 22).

The pro-apoptotic properties of GC are well established and are reviewed in Chapter II. Stress-induced CORT may be responsible for cell death in certain populations of cells in secondary lymphoid tissues. CORT may induce apoptosis via the upregulation of FAS or by other mechanisms such as the repression of survival factors.

Regardless of potential mechanisms involved in leukocyte death, very little remains known about the effect of stress and CORT on the trafficking of immune cells to and from secondary lymphoid tissues in the steady state. In order to fully understand the effects of stress on the outcome of a subsequent immune response, it is crucial to determine how neuroendocrine hormones modulate the steady-state “set point” that defines the context for how such immune responses will develop.
C. Conclusions

This dissertation presents evidence that DC are an important target of stress-induced GC. Proper DC function is critical for the efficient generation of an antiviral CD8$^+$ T cell response. When this response is compromised by stress, increased viral replication, delayed viral clearance, and exacerbated pathology can occur.

Our initial studies on the effects of CORT on DC function demonstrated that DC are susceptible to the effects of stress-associated CORT. From these studies were able to conclude:

1. CORT impairs the acquisition of a mature phenotype by BMDC in response to LPS stimulation (Fig. 2)

2. This impairment occurs with physiological concentrations of CORT, is mediated by GR, and does not occur via modulation of TLR4 or apoptosis (Figs. 3, 4).

3. CORT impairs the transcription of B7.1 and B7.2 and prevents the trafficking of MHC class II molecules to the plasma membrane in LPS-treated DC (Figs. 5, 6).

4. CORT prevents the LPS-induced downregulation of endocytosis by DC (Fig. 7).

5. CORT impairs DC for the production of pro-inflammatory cytokines in response to LPS stimulation (Fig. 8).

6. As a consequence of CORT-induced suppression of DC function, DC are less able to prime CD8$^+$ T cells (Fig. 9).

Once DC had been established as a target of CORT, we then determined that exposure to stress early in an infection impairs DC function in vivo. We also found
strong evidence that the effects of stress-induced CORT on DC lead to impaired CD8+ T cell responses that contribute to inefficient control of viral infection. The specific conclusions from these studies include:

7. Stress impairs the ability of DC from infected mice to induce the proliferation of antigen-specific CD8+ T cells (Fig. 19).

8. Exposure to stress early in an infection suppresses the generation of a subsequent CD8+ T cell response in a GR-dependent manner (Figs. 13, 14).

9. Stress-induced CORT acts on non-T cells to suppress the CD8+ T cell response (Figs. 17, 18A).

10. Early exposure to stress during infection results in increased viral load, delayed viral clearance and delayed resolution of viral lesions several days after stress is terminated (Figs. 12-14).

The findings presented in this dissertation illustrate the complexity of interactions that occur between the nervous, endocrine, and immune systems (Fig. 25). What may appear as a straightforward effect of stress-induced CORT on T cells is actually due, in part, to effects on other cells that are required for their efficient activation and function. As DC function is highly susceptible to CORT-mediated modulation, these cells stand out as an important target for stress-induced GC. It is important to realize that critical targets of GC (DC) are distinct from the cells in which immunosuppression is observed (T cells). If a therapy was designed that was aimed at enhancing T cell function by rendering T cells resistant to stress-induced GC, then it is likely that that strategy would fail. Beyond the implications within the field of neuroimmunology, the studies presented in this dissertation underline the importance of considering DC in any therapeutic
Figure 25. A model for stress-induced suppression of immunity to HSV. The perception of stress by the nervous system results in the release of GC into systemic circulation. These endocrine hormones act primarily on immune cells, including DC to suppress DC function in the generation of an antiviral immune response. DC are thought to be a principle target of stress-induced CORT, although CORT has a small effect on CD8$^+$ T cells themselves. Impaired DC function leads to impaired innate immunity and impaired CD8$^+$ T cell priming. Together, these effects on the immune system weaken the host’s ability to control HSV replication and pathology.
Stress

Nervous

Endocrine

Immune

CD8^+ T cell

HSV

DC

CORT

Figure 25
attempts to modulate T cell responses. Within the field of neuroimmunology, these studies provide further mechanistic evidence of the health consequences of chronic exposure to psychological stress and demonstrate the need for a greater understanding of the interactions that occur during stress between the nervous, endocrine, and immune systems to develop strategies that might prevent or alleviate stress-induced immunosuppression.
REFERENCES


120. Cumberbatch M, Kimber I. Dermal tumour necrosis factor-alpha induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. Immunology 1992;75(2):257-63.


Pooley JL, Heath WR, Shortman K. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol 2001;166(9):5327-30.


264. Ashcraft KA, Hunzeker J, Bonneau RH. Psychological stress impairs the local CD8+ T cell response to mucosal HSV-1 infection and allows for increased pathogenicity via a glucocorticoid receptor-mediated mechanism. Psychoneuroendocrinology 2008;33(7):951-63.


Curriculum Vitae
Michael D. Elftman

Education:
The Pennsylvania State University College of Medicine, Hershey, PA (2003-2009)
Degree: Ph.D. in Immunology and Infectious Disease (to be awarded December 2009)
Advisors: Robert H. Bonneau, Ph.D., Department of Microbiology & Immunology
M. E. Truckenmiller, Ph.D., Department of Microbiology & Immunology

Graduated Magna cum laude with B.S. in Microbiology from Lyman Briggs School
Advisor: Paul M. Coussens, Ph.D., Department of Animal Science

Honors and Awards:
Pennsylvania Department of Health – Graduate Research Supplement Award, 2007-2008
Selected as Pre-doctoral Trainee for Departmental NIH Training Grant, 2006-2007
Penn State College of Medicine – Karl H. Beyer, Jr., M.D., Ph.D. Scholarship, 2004
Michigan State University Department of Microbiology and Molecular Genetics – Gerhardt Award, 2003

Publications:


Selected Abstracts: