MODULATION OF THE NEONATAL T CELL-MEDIATED IMMUNE RESPONSE BY MATERNALLY DERIVED STRESS HORMONES

A Dissertation in
Cell and Molecular Biology
&
Molecular Medicine

by
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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

August 2008
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Abstract

A simple National Library of Medicine title search for the term “psychological stress” returns over 1400 titles, the majority of which address the impact of stress on the adult immune system. Despite this plethora of psychological stress-related publications, only a handful address the impact of stress on neonatal immunity. Neonates are vaccinated within days of delivery to generate an immune response that is maintained in some instances for the remainder of the individual’s life. Neonates begin to receive vaccinations while they are almost entirely dependent on their mother for nutrition and care. Maternally derived hormones, reflecting the mother’s psychological and physical conditions, are passed to the neonate via the breast milk. This passage could potentially expose the neonate to several immunomodulatory hormones, namely glucocorticoids and epinephrine. These hormones are known to hinder the adult immune response in the context of infection. The neonate is no exception, as exposure to maternally derived stress hormones hinders the primary T cell-mediated adaptive immune response to herpes simplex virus type-1 infection (HSV-1). Neonatal exposure to stress-like levels of glucocorticoids increased HSV-related morbidity and mortality via type II glucocorticoid receptor-dependent mechanisms. When examined ex vivo, this exposure 1) reduced overall splenic cellularity, 2) hindered the ability of CTL to expand clonally, 3) to express adequate levels of IL2Ra, 4) to lyse HSV-infected cells, and 5) and to produce pro-inflammatory cytokines. The hindrance of the primary immune response to HSV-1 infection is extended to the CTL memory repertoire. Moreover, these effects on the primary and memory HSV-specific T cell repertoires were reversed with the administration of a glucocorticoid receptor antagonist, which also prevented the HSV-related mortality. These findings demonstrate a role for glucocorticoids in the modulation of the neonatal immune system in response to infection.
Given that current neonatal immunization practices do not account for environmental factors at the time of immunization, these findings warrant an in-depth review of the clinical practices related to neonatal immunization. Moreover, these findings also shed light on the importance of managing post-partum maternal stress as exposure of the neonate to maternally derived hormones imprints the neonate’s immune system with both immediate and lifelong consequences.
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3. The body weight of neonates exposed to maternally derived corticosterone is indistinguishable from their vehicle counterparts 3 weeks after the removal of corticosterone.

4. HSV-specific T cells are detectable in the popliteal lymph nodes of neonates post-HSV-1 footpad infection (Yorty et al., 2004).

5. Maternally derived corticosterone increases HSV-1-associated pathogenicity in neonates.

6. Maternally derived corticosterone reduces neonatal survival post-HSV-1 infection.

7. The incidence of herpes simplex virus encephalitis is greater in neonates who were exposed to maternally derived corticosterone as compared to their vehicle counterparts.

8. The extent of edema, lymphocytic perivascular cuffing, and neuronophagia is far greater in neonates who were exposed to maternally derived corticosterone as compared to their vehicle counterparts.

9. Post HSV-1 infection, the survival of adult mice exposed to maternally derived corticosterone as neonates does not differ from their vehicle counterparts.

10. Glucocorticoid receptor antagonist mifepristone (RU486) is transferred from mother to neonate via the milk.
11. Neonatal exposure to RU486 along with corticosterone restores their survival post-HSV-1 infection to levels comparable to those observed in neonates exposed to vehicle.

12. Neonatal exposure to maternally derived corticosterone does not specifically target either CD4$^+$ or CD8$^+$ T cells. However, this exposure reduces the overall number of these cells in the spleen.

13. Neonatal exposure to RU486 along with corticosterone partially restores the overall numbers and percentages of CD4$^+$ and CD8$^+$ T cells in the spleen.

14. Neonatal exposure to maternally derived corticosterone reduces the proliferative ability of splenic-derived T cells.

15. Neonatal exposure to maternally derived corticosterone reduces the levels of IL-2 and its corresponding receptor, IL-2Rα.

16. Neonatal exposure to maternally derived corticosterone does not induce apoptosis in splenic-derived T cells.

17. Neonatal exposure to maternally derived corticosterone reduces the overall number and percentage of neonatal popliteal lymph node-derived HSV-specific T cells.

18. Neonatal exposure to RU486 along with corticosterone restores the overall number and percentage of neonatal popliteal lymph node-derived HSV-specific T cells.

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21. Neonatal exposure to maternally derived corticosterone reduces the overall number and percentage of neonatal popliteal lymph node-derived HSV-specific T cells that are also able to produce IFN-γ.

22. Neonatal exposure to RU486 along with corticosterone restores the overall number and percentage of neonatal popliteal lymph node-derived HSV-specific T cells that are also able to produce IFN-γ.

23. Corticosterone administered directly to the neonates via gavage elevates their serum corticosterone to levels comparable to those observed in neonates exposed to maternally derived corticosterone.

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25. Corticosterone administered directly to the neonates via gavage reduces the number and percentage of popliteal lymph node-derived HSV-1 specific T cells that were \( \text{CD8}^+gB_{498-505}^+ \).

26. Corticosterone administered directly to the neonates via gavage reduces the number and percentage of popliteal lymph node-derived HSV-1 specific T cells that were \( \text{CD8}^+\text{IFN-γ}^+ \).
27. Corticosterone administered directly to the neonates via gavage reduces the number and percentage of popliteal lymph node-derived HSV-1 specific T cells that were CD8⁻CD107⁺.

28. Maternally derived corticosterone does not alter the percentage of neonatal HSV-1-specific memory T cells.

29. Maternally derived corticosterone reduces the percentage of HSV-1-specific effector memory cells.

30. Exposure to both RU486 and maternally derived corticosterone restores the percentage of HSV-1-specific effector memory cells to levels observed in neonates exposed to vehicle alone.

31. Maternally derived corticosterone reduces the percentage of HSV-1-specific effector memory cells that are able to lyse their targets.

32. Exposure to both RU486 and maternally derived corticosterone restores the percentage of HSV-1-specific effector memory cells that are able to lyse their targets to levels observed in neonates exposed to vehicle alone.

33. Maternally derived corticosterone reduces the percentage of HSV-1-specific effector memory cells that are able to produce IFN-γ.

34. Exposure to both RU486 and maternally derived corticosterone restores the percentage of HSV-1-specific effector memory cells that are able to produce IFN-γ to levels observed in neonates exposed to vehicle alone.

35. Depleting CD8⁺ T cells from the neonate prior to infection enhances the survival of neonates exposed to maternally derived corticosterone but reduces the survival of neonates exposed to vehicle alone.
36. Unlike human neonates who retain maternal CTL received via the milk, maternally-derived CTL are not retained by C57BL/6 mice neonates.

37. The administration of epinephrine to the mother via the drinking water increases neonatal serum epinephrine levels.

38. Maternally derived epinephrine reduces neonatal splenic cellularity.


40. Exposure of neonates to a non-specific β adrenergic blocker (Nadolol) and maternal epinephrine partially restores their splenic cellularity.

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<tbody>
<tr>
<td>%</td>
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<tr>
<td>CpG</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g/L</td>
<td>Grams per Liter</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>gC</td>
<td>Glycoprotein C</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid Response Element</td>
</tr>
<tr>
<td>GRU</td>
<td>Glucocorticoid Regulatory Unit</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HBC</td>
<td>2-Hydroxypropyl-β-cyclodextrin</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>HSE</td>
<td>Herpes Simplex Virus Encephalitis</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes Simplex Virus type-1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus type-2</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-2R</td>
<td>Interleukin-2 Receptor</td>
</tr>
<tr>
<td>IL-2Rα</td>
<td>Interleukin-2 Receptor Alpha</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's-Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>Lysosomal Membrane Associated Protein-1</td>
</tr>
<tr>
<td>LAT</td>
<td>Latency Associated Transcript</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LF</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimoles</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MΦ</td>
<td>Macrophages</td>
</tr>
<tr>
<td>NBS</td>
<td>Newborn Bovine Serum</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post-Infection</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin Chlorophyll A Protein</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque Forming Units</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutin</td>
</tr>
<tr>
<td>PLN</td>
<td>Popliteal Lymph Nodes</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Leukocytes</td>
</tr>
<tr>
<td>PNS</td>
<td>Parasympathetic Nervous System</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular Nucleus</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>SIINFEKL</td>
<td>Serine-isoleucine-isoleucine-asparagine-phenylalanine-glutamic acid-lysine-leucine</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
</tr>
<tr>
<td>STAT4</td>
<td>Signal Transducer and Activators of Transcription 4</td>
</tr>
<tr>
<td>Tcm</td>
<td>Central Memory Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>Tem</td>
<td>Effector Memory Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Th</td>
<td>T Helper Cells</td>
</tr>
<tr>
<td>Th1</td>
<td>T Helper type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T Helper type 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>TNF-β</td>
<td>Tumor Necrosis Factor Beta</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/Volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/Volume</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
</tbody>
</table>
Acknowledgments

In the Name of Allah Most Gracious Most Merciful

First, I would like to thank the department chair that hosted me as a Cell and Molecular Biology student for five years, Dr. Richard Courtney, for his continued support professionalism and leadership style. I would also like to thank Dr. Henry Donahue for taking a chance on me. When I interviewed with him for graduate student candidate he said, “You are a little old for this”, nevertheless, he took a chance and offered me a position. I would also like to thank the entire faculty whom I have had the pleasure of meeting during my brief stay at the sweetest place on earth; your dedication and professionalism are reflected in your teaching methods, interaction with students, and the research performed in your laboratories.

A great deal of gratitude goes to my mentor Dr. Robert H. Bonneau. Over the past five years, Rob was not only a mentor but also a friend. The two roles intertwined in a fashion that supported both my scientific and professional development. The challenges Rob had to endure were countless, and as always challenging. Prior to joining his laboratory I worked in the healthcare field for over 10 years. I can honestly state that I have never met anyone as patient and professional as he is. Despite my continued efforts to empty his red pen while he was reviewing manuscripts for publication, he has never once given up or even made me feel the slightest bit of discomfort. I have learned a great deal from Rob, and I am sure our future collaborations will teach me even more. Rob thank you for being there every single time I needed you. Never once did you hesitate in offering a lending hand professionally and personally.
I would also like to thank Dr. Ralph Keil, not only for serving on my thesis committee but also for taking a genuine interest in my personal life. Dr. Keil is a distinguished educator; he taught genetics with a passion for science. His ideas and teachings were superb. Moreover, his insight on my thesis project were always valuable. Dr. Todd Schell’s scientific knowledge and never ending questions about my project have always challenged me to strive to achieve. I have often come up with answers to Dr. Schell’s questions days after he asked the, I guess I needed to relax and focus to answer his questions. His questions are ones I did not even dream about, and yet the answers have always served my professionally. I am grateful to have had Dr. Joan Summy-Long on my committee. She repeatedly provided a wealth of knowledge on how the different compounds I utilized could interact with each other, possibility resulting in a different conclusion. For all that and more, I would like to thank my committee members, who really guided this project from the first day they started serving on my committee.

Fellow laboratory members, we sailed this ship together through rough and calm waters. For not sinking this ship, I would like to thank Dr. Emmy Truckenmiller for her insightful political discussions and Dr. John Hunzeker for being a hands-on mentor, a friend who always managed to find humor in stories I shared with him about my kids, and his unique sense of humor that took me two years to see. Michael Elftman was always there for philosophical discussions, Kathleen Ashcraft made all of us work hard to match the mountains of data she brought to laboratory meetings, and Jennifer Mellinger for her superb technical skills and patience with last minute supply orders. I would also like to thank Dr. Aji Nair for his unique prospective on life and sense of humor.
No one has endured more than the love of my life, my lovely wife Azza. Her unconditional love, support and faith in my abilities contributed significantly to all my accomplishments. I am a better person for loving her and having her in my life. My daughter Dana, who defines a new meaning for logic and reasoning, was very understanding as when I relied on her to step up to the plate to fill the void I had left due to my late night experiments. My two boys, Shawqi and Adam, who gave a new meaning to life, were supportive in their own ways, they always wanted to go to school with daddy, they always wanted to help me review literature, and worked tirelessly on breaking my laptop. The boys taught me a new meaning for patience and provided me with a different prospective on life, a new meaning for love and endurance. For that and so much more, I am grateful for the wonderful family I am blessed with. Nothing shook the foundation of this family not a tornado, a premature delivery, a terminal illness, late night experiments, or repeated overseas trips because we simply loved each other.

I cannot find words to describe how grateful I am to the man who taught me everything I need to know in this life, my dad. His wealth of knowledge makes me feel small in front of him, and always inspired me to excel in life. When I was a young boy, he used to drive me around town and quiz me about automobiles make and model. I never understood the importance of this until I started reading about how early childhood memory development techniques imprinted learning ability throughout life. Even though I researched the impact of stress on immunity for the past five years, he still knows more than I ever will about this subject, and many others. Finally, when I visited him he said “the way you think has changed, you became more critical and subjective”, it is like he was awarding me a degree of his own. In achieving this level of scientific
accomplishment I am fulfilling one of my mother’s lifelong dreams; for having believed in me and supported me throughout the years, and many more to come, I am eternally grateful. My two sisters always believed and supported me, even though when we were kids and I used to convince them to bury eggs and an egg tree will grow. For their love and faith in me I thank them from the bottom of my heart. Last but not least, my friends who stood by me and supported me throughout these years, Dr. Jamal Joudeh, Dr. Ammar Al-Laham, and my cousin Waeil I thank each and every one of you and for your support.
In the Name of Allah Most Gracious Most Merciful
Introduction

Over the past decade, research in our laboratory focused on the interaction between the nervous, endocrine, and immune systems. Each of these systems is capable of up- and/or down-regulation of the other depending on the physical and psychological state of the host. This psychoneuroendocrine interaction is of great importance, especially in light of several key discoveries dating back to Hans Selye’s definition of psychological stress in 1947. The ability of psychological stress to activate the hypothalamic-pituitary-adrenal axis (HPA) culminates in increased synthesis and release of several immunomodulatory hormones, namely glucocorticoids (cortisol in humans, and corticosterone in rodents). The increased levels of circulating glucocorticoids result in a wide array of metabolic and immunologic alterations in an attempt to restore homeostasis.

During the first few weeks of life, human neonates are almost entirely dependent on their mother’s milk for nutrition and immunity. This dependence comes at the cost of being exposed to maternally derived hormones that are passed to the neonate via the milk. These hormones reflect the mother’s physical and psychological states. Thus, the exposure to stress-like levels of maternally derived hormones, in addition to hormones the neonate is able to produce in response to stimuli, further elevates the levels in their circulation, consequently magnifying the effects of these hormones. It has been well documented that human neonates are capable of responding to stress as measured by the activation of their HPA axis. Neonatal response to psychological stress leads to increased levels of circulating glucocorticoids. Moreover, neonates that nurse from mothers
exposed to psychological stress are also exposed to their mother’s increased glucocorticoids levels. These hormones, amongst others, are well known to cause modulation in the immune response in adults (Glaser and Kiecolt-Glaser, 2005b). Therefore, we hypothesized that neonatal exposure to these elevated levels would also result in neonatal T cell adaptive immunomodulation. This hypothesis has great clinical implications as neonates receive a variety of vaccines and are exposed to a variety of pathogens during the first few months of life, beginning as early as a few days post-delivery. The immune response to these vaccinations is assumed to provide lifelong immunity against pathogens as immunological memory of some antibodies against childhood vaccinations has been detected 75 years later (Gourley et al., 2004). However, if at the time of vaccination the immune system was hindered as a result of the presence of elevated levels of glucocorticoids, maternally driven or generated by the neonates in response to psychological stress, the immune response to these vaccinations may be suboptimal, at best. This assumption is supported by findings demonstrating that medical students undergoing stressful examinations are hindered in their ability to respond to hepatitis B vaccination (Glaser et al., 1992). Moreover, it has been demonstrated in children, that breastfeeding shortly prior to vaccination amplifies the immune responses generated against vaccines (Pabst et al., 1989; Pabst et al., 1997). It has also been reported that even years after discontinuing breastfeeding, children who were breastfed for more than 90 days as neonates demonstrated a more potent response to childhood vaccination (Silfverdal et al., 2007). These findings further emphasize the importance of neuro-endocrine-immune interaction and the clinical implications of our studies.
In the studies described herein, neonatal exposure to stress-like levels of glucocorticoids, maternally derived or administered directly to the neonate, increased HSV-associated morbidity and mortality. When examined postmortem, approximately 70% of the HSV-infected neonates who were previously exposed to stress-like levels of glucocorticoids exhibited brainstem edema, lymphocytic perivascular cuffing, and neuronophagia. It has been documented that brainstem edema is the number one cause of HSV-associated mortality (Mori et al., 2005). This increased HSV-associated morbidity and mortality was the clinical manifestation of an impaired immune system, of particular interest to our studies, T cell-adaptive immune responses. The ability of CD8<sup>+</sup> T lymphocytes to attain their lytic function and expand clonally is critical in the battle against invading pathogens. When examined ex vivo, the percentage of HSV-specific popliteal lymph node-derived T cells was significantly reduced in the neonates who were exposed to stress-like levels of glucocorticoids. This reduction was not attributed to apoptosis but rather attributed to the fact that these cells produced less interleukin 2 (IL-2, a necessary interleukin for proliferation). Additionally, the expression levels of IL-2 receptor alpha (IL-2Rα) on their cell surface was also significantly reduced, thus, further reducing their ability to respond to proliferative signals. Additional analysis of these HSV-specific T cells revealed a reduction in the number and percentage of cytotoxic T lymphocytes (CTL) that are able to produce proinflammatory cytokines (namely interferon gamma, IFN-γ) and also a reduction in the number of cells that were able to lyse HSV-infected cells. Interestingly, these effects were mediated by the interaction between glucocorticoid and their type II receptor as the administration of the receptor
antagonist mifepristone (RU486) restored survival, which is a reflection of a “healthy” T cell adaptive immune response.

The ability of the immune system to retain a memory T cell repertoire is essential to further reduce the time needed to respond to a secondary challenge with a pathogen it has previously encountered. This repertoire results from the contraction of the primary pathogen-specific T cells as a result of apoptosis. One could hypothesize that if the primary immune response to HSV infection were impaired subsequent to the exposure of stress-like levels of glucocorticoids, the memory pathogen-specific repertoire would also be impaired. Indeed, evidence presented herein clearly demonstrates a hindered pathogen-specific T cell repertoire as a direct consequence of exposure to stress-like levels of glucocorticoids. This hindrance is perpetuated via type II glucocorticoid receptor mediated mechanisms as the administration of a glucocorticoid receptor antagonist completely restored the functional capacity of this memory pathogen-specific repertoire.

Glucocorticoids are not the only immunomodulatory hormone produced in response to stress. The sympathetic nervous system is also activated in response to stress, thus, leading to increased synthesis and release of norepinephrine at the nerve synapses and epinephrine systematically. Several publications describe the sympathetic nervous system response to psychological stress in both human and animal models (Altemus et al., 2001; Antoni, 2003; Leo and Bonneau, 2000a, b; Leo et al., 1998; McCarty et al., 1991). Since glucocorticoids are elevated in response to psychological stress, and immunomodulation occurs under such conditions, one could hypothesize that other hormones that are elevated in response to psychological stress could also impact the
immune state of the host. For example, catecholamines are also elevated during psychological stress, these hormones are also known for their ability to modulate metabolic and immune responses. Evidence supporting this hypothesis in adult animal experimental models has been reported (Altemus et al., 2001; Leo and Bonneau, 2000a; Webster et al., 2002). Furthermore, to extend these findings, we demonstrate in the studies described herein that elevated levels of catecholamines, namely epinephrine, are transferred from mother to neonate via the milk. Moreover, this increased level of epinephrine results in a reduced neonatal splenic cellularity, and increased HSV-associated morbidity and mortality. The issue is far more complicated than just a cause and effect relationship, as simply blocking the β adrenergic receptor using nadolol does not fully restore splenic cellularity further indicating that splenic cellularity is governed by multiple factors. Interestingly, administering the β adrenergic receptor antagonist increased the survival of HSV-infected neonates who were also exposed to elevated levels of epinephrine to levels comparable to their counterparts exposed to nadolol alone. However, the administration of nadolol alone decreased neonatal HSV-associated survival clearly demonstrating that sympathetic nervous system signaling is critical for generating an adequate immune response to HSV infection.

The effects of glucocorticoids extend beyond the immune system to affect adrenal medullary cells (chromaffin cells) as exposure of these cells to elevated levels of glucocorticoids resulted in the increased synthesis and release of catecholamines (Kelner and Pollard, 1985). This increase in the concentration of circulating catecholamines is further confirmed by our studies. When we administered a glucocorticoid receptor antagonist we were unable to completely reverse the reduction in the splenic cellularity
that resulted from exposure to elevated levels of glucocorticoids. This partial restoration does not only confirm a role for glucocorticoids in controlling splenic cellularity, but also implicates other stress hormones, namely catecholamines, in determining the cellularity of this major secondary lymphoid organ. Exposure to β-adrenergic receptor antagonist and stress-like levels of glucocorticoids also partially restored splenic cellularity, further confirming a role for catecholamines in determining splenic cellularity. Only after the combined administration of both a glucocorticoid receptor and a β-adrenergic receptor antagonist were we able to fully restore splenic cellularity subsequent to the exposure of stress-like levels of glucocorticoids.

Evidence described herein sheds some light on the maternal influence on the neonatal T cell adaptive immune system. Moreover, these findings warrant further examination of clinical practices employed postpartum. Management of mothers during the postpartum period benefits not only the mother but also the neonate as prolonged exposure to maternally derived stress hormones imprints the neonate’s nervous, endocrine, and immune systems. The devastating impact of maternal stress, and the subsequent exposure of the neonate to increased levels of stress hormones, has been shown to have life-long effects the neonates in both human (Coussons-Read et al., 2005) and animal models (Catalani et al., 2000). Additionally, these findings further emphasize the need for better neonatal vaccination strategies as neonates subjected to elevated levels of stress hormones are not able to mount an effective immune response to vaccination (Jackson and Nazar, 2006). This optimal and protective immunity generated in response to childhood vaccinations is assumed to be present, and for some vaccines, is also assumed to protect the individual for the remainder of their life. Given that this
assumption is not entirely valid, a detailed assessment of both the mother’s and neonate’s psychological status needs to be documented prior to vaccination.
Chapter I: Literature Review

A. Immune system

1. Introduction

Naive T lymphocytes are the predominant cell type in the absence of infection. T lymphocytes are divided into two major groups based on their cell surface marker expression. Cells that express the CD4 molecule are known as CD4⁺ cells, while cells expressing the CD8 molecule are known as CD8⁺ cells. The fate of naive T lymphocytes upon exposure to antigen is governed by the cytokine milieu at the time of exposure (Janeway, 2008). T cells become activated when they recognize antigen presented in the context of MHC on the surface of professional antigen presenting cells (APC). The main goal for activated T lymphocytes is to eliminate the pathogen infected-cells. How that is achieved is best described as a well coordinated response to ensure maximum efficiency and minimal self-damage.

The cellular components of the adaptive immune system include CD4⁺ and CD8⁺ T lymphocytes. Upon activation, CD4⁺ T lymphocytes are capable of differentiating into T helper type 1 (Th1) or T helper Type 2 (Th2) subsets depending on the cytokines available at the time of differentiation. Interleukin-12 (IL-12) produced by APC acts in concert with signal transducers and activators of transcription 4 (STAT4) and T-bet to guide the CD4 proliferation response down the Th1 pathway. The hallmark cytokines for Th1 cells are IFN-γ, tumor necrosis factor beta (TNF-β) and IL-2. On the other hand,
when interleukin-4 (IL-4) is produced by APC, and through STAT6 signaling and GATA-3 action, activated CD4$^+$ T lymphocytes follow the Th2 pathway. The most abundant cytokines produced by Th2 cells are IL-4, IL-5, IL-10, IL-13, and TGF-β (Janeway, 2005; Seder and Ahmed, 2003). The cytokines produced by each of the subsets suppresses the development of the alternate response (i.e. IFN-γ suppresses the Th2 response, and similarly, IL-10 and TGF-β suppress the Th1 response). Although a full immunologic response can be attained in the absence of CD4$^+$ T lymphocytes, the quality of the response is adversely impacted under these circumstances. The cytokines produced by the differentiated CD4$^+$ T lymphocytes serve specific functions during the adaptive immune response. IFN-γ, for example, activates macrophages leading to pathogen clearance through a variety of mechanisms. Additionally, through their CD40 ligand receptor (CD40L), CD4$^+$ T lymphocytes, imprint a “unique molecular signature” on cells bearing the CD40 receptor thus enhancing their function (Bourgeois et al., 2002). For example, CD8$^+$ T lymphocytes have an improved memory response when developed in the presence of CD4$^+$ T lymphocytes (Janeway, 2008).

CD8$^+$ T lymphocytes are a critical part of the adaptive immune response. Unlike CD4$^+$ T lymphocytes, CD8$^+$ T lymphocytes are capable of direct killing through cytolysis. Activated CD8$^+$ T lymphocytes are capable of recognizing cells infected with the pathogen to which they have been primed. Through direct interaction with infected cells, activated CD8$^+$ T lymphocytes are able to release the contents of cytolytic granules to induce apoptosis in their targets as discussed elsewhere in this literature review. Additionally, CD8$^+$ T lymphocytes are capable of mediating effector function through cytokine production, particularly IFN-γ.
One of the hallmarks of the adaptive immune system is the ability to efficiently mount an antibody response against pathogens. This response depends on the ability of B lymphocytes to generate such specific and efficient antibodies. B lymphocytes are activated via T cell-dependent or T cell-independent mechanisms. In both pathways B lymphocytes receive the first activation signal when the antigen interacts with the cell surface receptor. However, in the T cell-dependent pathway a second signal is delivered by the CD4+ T lymphocyte that recognizes the major histocompatibility complex II:antigen complex (MHC-II:Ag complex) displayed on the surface of the B lymphocyte. Subsequent interactions between the CD40L on the surface of CD4+ and the CD40 receptor on the surface of B cells, in addition to the cytokines produced by the CD4+ T lymphocyte and their respective receptors on the surface of the B lymphocyte, result in full activation of the B cell in a T cell-dependent fashion. The antigen itself or other non-thymus derived cells deliver the second signal needed for B cell activation in the T cell independent pathway. Activated B lymphocytes produce antibodies specific against the pathogen they were primed against. These antibodies bind to the pathogen leading to the activation of the complement cascade, eventually leading to pathogen clearance (Bourgeois et al., 2002; Carsetti, 2004; Harnett et al., 2005; Janeway, 2005).
2. **Branches of the immune system**

a. **Innate immunity**

The orchestrated interplay between the innate and adaptive arms of the immune system ensures 1) a proper response to the pathogen and 2) tolerance to self-antigens. The innate components of the immune system are thought to predate the adaptive one because it recognizes self- and non-self pathogens more efficiently and it exists in all multi-cellular organisms, vertebrates and non-vertebrates alike (Medzhitov and Janeway, 1997). Over several million years, the innate immune system became efficient in eliminating pathogens without causing self-damage. The innate system is able to achieve this goal by recognizing essential molecular patterns that are not expressed on the host’s cells but are shared by many pathogens (Medzhitov and Janeway, 1997). Pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (Gram-negative bacteria), teichoic acid (Gram-positive bacteria), un-methylated CpG motifs (bacterial DNA), double-stranded RNA (some viruses), and mannans (yeast) are examples of PAMPs recognized by the innate immune system (Hauschildt and Kleine, 1995; Krieg et al., 1995; Lampson et al., 1967). Non-clonal PAMP receptors enable the host to recognize a variety of pathogens that share these patterns. These receptors comprise several protein families and are known as pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997). These receptors (PRRs) are expressed on a variety of cells including natural killer cells, neutrophils, and macrophages. Recognition of
pathogens by the PRRs activate certain components of the innate immune system, ultimately resulting in pathogen clearance.

Complement is another component of the innate immune system that ultimately results in pathogen clearance. Complement is a set of plasma proteins working in concert to clear pathogens. In addition to the pathogen’s ability to directly activate the complement cascade, pathogens bound to antibodies are able to indirectly activate the same system promoting pathogen clearance via phagocytosis or direct pathogen lysis (Janeway, 2005).

b. Adaptive immunity

The naturally attenuated neonatal adaptive immune response is attributed to several factors including the lack of antigen exposure needed to develop receptor diversity and the lack of appropriate cellular components essential for such a response. In rodent models for example, the percent of dendritic cells and lymphocytes in neonates does not reach adult levels until about five weeks of age (Dakic et al., 2004). Thus, the inability to present antigens to naive T lymphocytes impairs the activation and generation of antigen-specific T lymphocytes. These cells are an important component of the adaptive immune system. Consequently, the innate immune system is predominantly responsible for protection against pathogens for the first few days of a newborn’s life. During this time, the neonate is almost entirely dependent on the mother’s milk for passive protection and immune-system education. The mammary gland is complex, it provides nutrition and immunity to the nursing infant (Sordillo et al., 1997). Antibodies passively passed
through the milk to the neonate helps to protect the neonate until the neonate’s immune system is competent enough to mount its own response (Yorty and Bonneau, 2004b). It is believed that one of the mechanisms responsible for the neonate’s immune system’s education of self- versus non-self antigen is the passage of suppressive chemokines through the mother’s milk (Kelly and Coutts, 2000; Kovarik and Siegrist, 1998). This passage of chemokines ensures proper immune system development that could respond to pathogen challenge in a balanced fashion (Kelly and Coutts, 2000; Kovarik and Siegrist, 1998).

i. The importance of T cell-mediated adaptive immunity

Antibodies, the major component of the humoral adaptive immune system, do not gain access to viruses that spread from cell-to-cell; therefore, T cell-mediated immune response is critical in the elimination of virus-infected cells. The precursor frequency of naive antigen-specific CD8⁺ cells ranges from 1 in ~10,000 to 1 in 100,000 (Blattman et al., 2002; Nugent et al., 1994). However, activation signals received by these pathogen-specific naive CD8⁺ T cells from APC induces proliferation. One naive pathogen-specific precursor could give rise to more than 10,000 antigen-specific daughter cells over 13 divisions, a process that could take anywhere from 5 to 8 days (Harty and Badovinac, 2008). This proliferation gives rise to a population of cells known as effector T cells or CTL. These cells leave the lymphoid organs to survey the body. Upon encountering a pathogen-infected cell that they can recognize, CTL perform a wide array of antimicrobial functions that include lysing the infected cells and releasing cytokines to
further magnify the anti-inflammatory response. These measures deprive the virus the opportunity to propagate.

**ii. Cytolytic granule-mediated apoptosis**

Activated CTL contain cytolytic granules that induce apoptosis of the target cell when released. These cytolytic granules (lysosomes) contain, among other enzymes, perforin and granzymes B. Perforin, a membrane-perturbing protein that disrupts the integrity of the cytoplasmic membrane of virus-infected target by forming transmembrane channels, allows entry of granzymes B. Granzyme B is a serine protease with irreversible roles causing caspase-dependent and -independent apoptosis (Kagi et al., 1994; van den Broek et al., 1996). The release of the lysosomal contents involves transient expression of the lysosomal-associated membrane protein-1 (LAMP-1) on the plasma membrane that can be monitored using fluorescently-labeled antibodies.

**3. Memory immune repertoire**

The memory immune response is necessary for long-term immune protection, both for naturally- and vaccine-generated immune responses. While the exact lineage of memory T cell subsets is still under debate in the scientific community, there is a consensus that the elimination via apoptosis of up to 95% of effector CTL generated during the primary response leaves behind a long-lived population of cells known as memory CTL (CTLm). Thus, increasing the frequency of pathogen-specific precursor
from ~1 in 100,000 (described above) further reduces the time needed to respond to a secondary pathogenic encounter. In humans, CTLm have been detected 75 years post-vaccination, inferring a lifetime protection (Gourley et al., 2004). Two distinct subpopulations have been identified in the CTLm repertoire, central CTLm (Tcm) and effector CTLm (Tem). What remains subject to scientific debate is the origin of these two subpopulations and the role each one plays upon secondary pathogenic encounter. The branched model theory, which proposes that Tcm and Tem are two distinct subpopulations generated as a result of the primary cell’s contraction is supported by evidence that both Tcm and Tem after the aforementioned contraction. On the other hand, the linear model theory proposes that effector CTL retract leaving behind Tcm that could give rise to Tem with or without secondary pathogenic encounter. While, our studies do not focus on the origin of these two subpopulations, rather, we examined the impact of stress hormones on these subpopulations in neonatal C57BL/6 mice.

B. Herpes simplex virus (HSV)

1. Introduction

With over 100 viruses in the Herpesviridae family, the range of disease these viruses cause ranges from mild cosmetic irritants to life-threatening illnesses. Herpesviridae viruses are grouped into three subfamilies (α, β, and γ) based on their biological and physical properties. One of the most intriguing characteristics of these
viruses is their ability to establish latency in the host. During this latency period, which can extend to years and decades in some instances, the host remains asymptomatic. The real mystery is the conditions that are required for the reactivation these viruses. Several studies have outlined a variety of factors, all of which involve stress. However, no single determinant factor has been determined in the reactivation process (Bonneau and Hunzeker, 2007).

2. The Virus

a. Structure

HSV is an enveloped DNA virus that replicates in the nucleus (Flint et al., 2000). This genome can “isomerize” or recombine via inverted repeat sequences. At least 84 open reading frames have been identified in the HSV-1 genome. The nucleocapsid surrounding this DNA genome is a lipid bilayer measuring 100-110 nm in diameter and is composed of six major proteins. The icosahedral nucleocapsid of Herpesviridae viruses contains a DNA genome organized into a 126- Kilobase (Kb) long region and a 26-kb short region. The surface of this nucleocapsid is organized using VP5 (150 KDa major nucleocapsid proteins) on the faces of a T=16 icosahedron composed of 162 capsomeres arranged as 150 hexamers (Flint et al., 2000). Surrounding the nucleocapsid is a thick proteinaceous layer called “tegument”. The tegument contains a continuously growing number of virally encoded proteins recently documented to range from 15-20 proteins (Abaitua and O'Hare, 2008; O'Regan et al., 2007). One of these proteins, VP1-2 encoded
by UL36, is essential for productive infection (Abaitua and O'Hare, 2008; O'Regan et al., 2007). Finally, surrounding the tegument is a host-derived lipid bilayer studded with virally encoded glycoproteins necessary for virus entry. These glycoproteins are normally designated as gB, gC, gD, gE, gF, gG, and gX. Of particular interest to our research is gB as it contains the HSV-1 immunodominant sequence gB{498-505} (Bonneau et al., 1993b). When this immunodominant sequence is conjugated to fluorescent dye, we are able to detect the presence of the peptide-dye complex using flow cytometry analysis.

b. Life Cycle of HSV

HSV utilizes the cellular transcription machinery under the control of viral promoters during the productive infection phase of its life cycle. Locally infected epithelia cells shed the virus; some of which enters sensory neuron terminals and travels anterogradely to the neuronal nucleus where it establishes latency (primarily the trigeminal ganglia for HSV-1) (Knipe and Cliffe, 2008; Peng et al., 2008). Periodic reactivation can occur during the host’s life cycle. Several factors result in this reactivation event, one of which is psychological stress. Although some neurons are destroyed during reactivation, most neurons are refractory to this event. During reactivation, the virus travels retrogradely along the axons and is released to infect epithelia cells often at the original site of entry. The most common symptom associated with HSV-1 reactivation is oral/facial lesions. Serious complications, often associated with high mortality, such as disseminated HSV infection and viral encephalitis can also occur (Lachmann, 2003).
c. **Etiology**

The ability of HSV to travel retrogradely to the brain via neurons enables it to establish infection in the brain resulting in herpes simplex encephalitis (HSE). This form of encephalitis accounts for 10-20% of all viral encephalitis cases. In adults and older children (> 6 months) the prevalence of this form of encephalitis is one in 250,000-500,000 individuals with mortality as high as 60% even with aggressive treatment (Aurelian, 2005). Survivors cope with permanent, severe, and often debilitating neurological damage (Aurelian, 2005). Neonates born to HSV-infected mothers are potentially bathed in HSV-infected fluids during a natural delivery. This poses a great risk for the neonate for contracting HSV during delivery. Not surprisingly, 26 out of 100,000 live births to mothers who are HSV-1 seropositive are infected with HSV-1 (Brown et al., 2003).

d. **Prevention and treatment**

Fortunately, HSV is only transmitted through direct intimate contact. Therefore, prevention is accomplished by simply avoiding direct intimate contact with HSV-infected individuals. Regrettably, infected individuals are often asymptomatic and they are unrecognized; therefore, protection should always be used during intimate contacts, at least in non-monogamous relationships. Neonates are at a great disadvantage as they are exposed to HSV prenatally, during delivery, and postnatally. Natural delivery significantly increases the risk of contracting HSV for the neonate. Five percent of
neonates delivered to women from whom HSV was isolated at the time of labor were also infected with HSV. Recent examination of the incident rate of neonatal HSV infection concluded that caesarean delivery reduced the risk of infection from HSV-infected mothers from 7.7% for vaginal delivery to 1.2% (Brown et al., 2003). Adequate treatment for α-herpes virus infection in immunocompetent host includes acyclovir, penciclovir, and brivudin. Both acyclovir and penciclovir are considered acyclic nucleoside analogues (De Clercq, 2004; Martinez et al., 2008). In neonates, high doses of acyclovir (60 mg/kg/day for three weeks) have been successfully used to treat HSV infection (Melichar et al., 2004).

3. Host response to HSV infection

a. Introduction

The ability of the host to overcome HSV infection relies heavily on the two arms of the immune system, innate and adaptive. The coordinated immune response to HSV is the result of stage dependent and independent events. The response begins with the containment phase (innate immunity), and ends with the curative phase (adaptive immunity) (Kohl, 1989). Although complete eradication of the virus is an impossible mission, controlling the virus in its non-replicative latent stage is achievable. The main goal of the containment phase is preventing the spread of virus particles either via cell-to-cell spread or replication and release for new infection. The containment phase is initiated quickly after viral entry and can last up to two weeks (Kohl, 1989). At this
phase, three types of cells rush to the site of insult. Two phagocytic cell types which are polymorphonuclear leukocytes (PMNs) and macrophages (MΦ); these cells are able to directly engulf and destroy the virus through the production of interferon. Upon encountering HSV particles, PMNs and MΦ produce alpha and beta interferons. Both of these interferons render normally HSV-susceptible cells resistant to viral infection (Kohl, 1989). The third cell type that rushes to the site of insult are natural killer cells (NK). These cells are activated by lymphokines such as interferon or IL-2. Once activated, NKs proliferate and selectively destroy HSV-infected cells (Kohl, 1989). As the containment phase continues, the curative phase (adaptive immunity) is ramping.

The curative phase begins with antigen processing and presentation by none other than professional APC. These cells also produce cytokines IL-1 and TNF. The combination of presented antigen and cytokines leads to the activation of a variety of lymphocytes (B and T). B lymphocytes, when activated, are able to produce antibodies. Antibodies that recognize the virus and neutralize its ability to enter host cells are known as neutralizing antibodies. The other class of antibodies produced by activated B lymphocytes recognize and adheres to infected cells marking these cells for destruction via a process known as antibody-dependent cellular cytotoxicity (ADCC); (Kohl et al., 1990; Landers et al., 1994). The interplay between the two types of antibodies produced by B lymphocytes has been shown, in C57BL/6 mouse models, to be protective in neonates exposed to stress-like levels of glucocorticoids either pre- or post-natally (Yorty and Bonneau, 2004b).
b. **T Cell Activation**

The majority of T lymphocytes are naive and are unable to recognize and eliminate infected cells. A critical event has to take place for these naive cells to be activated and become “licensed” and acquire their anti-microbial functions; namely activation by a professional APC. These APC, in their immature state, are considered the “sentinels” of the immune system. APC continually sample their environment in search of antigens. Upon encountering an antigen, APC become activated and are able to process the antigens and present 8-10 amino acid-long peptides derived from these antigens in the context of their MHC-I complex. Moreover, activated APC express high levels of co-stimulatory molecules (B7, 4-IBBL) because of their activation. Naive T cells, on the other hand, are able to recognize this peptide:MHC class I complex on the surface of these APC. In addition to the primary signal received through the MHC class I, CD8$^+$ T cells require additional co-stimulatory signals. 4-IBBL and CD40L on the surface of the APC interact with 4-IBB and CD40 on the surface of CD8$^+$ T cells providing the necessary “licensing” signals for CD8$^+$ T cells. This licensing, or activation, of the naive CD8$^+$ T cell by APC results in a CTL that is able to recognize infected cells, produce pro-inflammatory cytokines, and lyse its target-infected cell (Janeway, 2008).
c. **Neonate**

i. **Innate immunity**

The innate and adaptive immune systems work synchronously to ensure host survival and well-being. It is believed that the innate system not only controls the pathogen, but also initiates the adaptive arm of the immune system (Jullien et al., 1997). More specifically, exposure of the neonate to HSV during vaginal delivery is more prolonged and disseminated when compared to adults and child encounter with the virus, which is usually brief and restricted (genitalia, eye, or lip); (Jullien et al., 1997). During vaginal delivery, the neonate may spend hours in the birth canal immersed in HSV-infected fluid with virus titer ranging from $10^6$ to $10^8$ pfu/mL (Kohl, 1989; Kohl et al., 1990). During this time, the virus is in direct contact with the fetus’s skin, mucosal membranes, and even lung resulting in a heavy inoculation of these sites. Several studies focusing on differences between the neonatal and adult immune responses to HSV have eloquently elucidated that the neonatal shortfalls are in both the containment and curative phases (Kohl, 1989; Kohl et al., 1990; Levy et al., 1999). The first two components of the containment phase are PMNs and MΦ. These two cell types are considered non-permissive for HSV replication in adults. However, neonatal alveolar MΦ demonstrate increased viral replication when compared to adult MΦ (Hayward et al., 1988). This is not a defect in the cells since they become activated in response to mitogen or anti-CD3 antibody in vitro. As discussed earlier, one of the hallmarks of the containment phase is the production of interferon and TNF by PMNs and MΦ. Interferons protect cells from
viral infection, activate NK cells, MΦ, and help lymphocyte function during the containment phase (Kohl, 1989; Kohl et al., 1981; Kohl et al., 1990).

The third component in the containment phase is NK cells. As discussed earlier, these cells are activated by interferons and lymphokines (IL-2 in particular). In vitro studies demonstrated that neonatal NK cells are less responsive to IL-2 stimulation when compared to adult cells (Kohl et al., 1981). Not only are the neonatal cells less responsive, but also the magnitude of the response and the total number of cells responding, is lower in neonates than in adults.

ii. Adaptive immunity

As the human neonate reaches five weeks of age, the composition of its T lymphocyte population becomes indistinguishable from that of an adult. During this time, the neonate is exposed to increasing numbers of foreign pathogens resulting in the development of a T lymphocyte repertoire that recognizes and is able to quickly respond to stimuli when re-challenged. Increased exposure to foreign antigen alone is not sufficient for the development of a healthy adaptive immune system. The number of APC also increases within the first five weeks until it reaches adult levels (Dakic et al., 2004). Like in adults, these cells are needed to present antigen to the T lymphocytes, a critical step in the T lymphocyte activation process. Maternal factors also play a critical role in the neonate’s immune development process. Components passively passed through the mother’s milk include chemokines and cellular subsets. These factors assist in the maturation of the neonate’s adaptive immune system by providing necessary
signals needed not only for T lymphocyte activation and maturation, but also for immune response control, thus, evading potential over-reactive immunity complications.

The majority of the T lymphocytes in human neonates up to five weeks of age are CD45RA+ (naive phenotype); (Kovarik and Siegrist, 1998). With increasing exposure to foreign antigen, the neonate’s T lymphocytes become activated and the cells begin to acquire the CD45RO+ mature/memory phenotype (Bertotto et al., 1990; Kovarik and Siegrist, 1998). Upon activation, neonatal T lymphocytes become more sensitive to activation as the threshold of their T cell receptor decreases. *Ex vivo* testing established that neonatal T lymphocytes have the normal functions adult T lymphocytes have but their activation threshold level is altered (Kilpinen et al., 1996; Whary et al., 1995). Additionally, the neutral immune response in neonates is biased towards Th2. It is not clear if this bias is a result of 1) the abundance of Th2 cytokines circulating in the mother’s blood during pregnancy; 2) the lack of necessary APC; 3) a combination of these factors; or 4) others factors (Al-Shammri et al., 2004; Zhang et al., 2004). The mere presence of IL-4 (one of the Th2 cytokines) at the time of T lymphocyte activation is sufficient to inhibit IL-12Rβ2 chain expression, thus forcing the cells towards a Th2 response (Delespesse et al., 1997; Ohshima and Delespesse, 1997). It is worth noting that these data do not contradict the fact that neonates are able to generate an effective response to vaccines received immediately after delivery (e.g. hepatitis B) due to the fact that such immunization use Th1-driving adjuvants, or are made of live-attenuated agents (Barrios et al., 1996a; Barrios et al., 1996b; Forsthuber et al., 1996).

The second arm of the adaptive immune response is the ability to produce antibodies by B lymphocytes against the foreign pathogen. Maternal IgG crosses the
placental barriers into the fetus’s bloodstream providing a primitive yet effective mechanism to control infection. Additionally, SIgA passively passed through the milk for additional protection (Hanson et al., 1990; Shore et al., 1977). It is worth noting that newborn infants are able to produce IgM, IgG, and IgA upon exposure to foreign antigens. However, these antibodies do not reach adult levels until about six month of age. It is believed that transplacental and transmammary anti-idiotypic antibodies prime the immune system making it better suited to respond to antigenic challenges (Hanson et al., 1990).

Several published studies focused on the curative-phase response for neonates. The first aspect of the adaptive response is the production of antibodies, neutralizing and ADCC. Since neonatal HSV infection is transmitted from mother to infant, the infection takes place in an environment where anti-HSV-antibodies already exist and are transmitted to the neonate either from across the placenta or via the mother’s milk (Kohl, 1989; Kohl and Loo, 1984; Yorty and Bonneau, 2004b). However, the primary neonatal antibody response is delayed in neonates when compared to adults (Adkins, 2005; Kohl et al., 1986; Koyama and Kasahara, 1975). In published studies, only 19% of human neonates demonstrated a 4-fold rise in antibody titer of neutralizing antibodies in the first month post-infection (Sullender et al., 1987). The main effective antibody type in controlling infection in neonates is passively transferred to the neonate either in the womb or via the mother’s milk (Yorty and Bonneau, 2003, 2004b). Additionally the ability of human cord blood leukocytes to adhere to antibody-coated infected cells (ADCC reaction) is significantly less than adult leukocytes (Kohl et al., 1984).
The response to HSV infection in neonates is dampened when compared to children and adult responses (Pass et al., 1981; Sullender et al., 1987). Specifically, only one third of neonatal T lymphocytes respond to HSV infection when compared to adults. However, the T lymphocyte population responds to \textit{in vitro} mitogen stimulation ruling out generalized deficiency in proliferation ability (Kohl, 1989). Additionally, when compared to adult T lymphocytes, neonatal cells demonstrate a lag in interferon gamma production but comparable IL-2 production (Burchett, 1987; Kohl, 1989). In summary, neonates are able to respond to HSV infection with some time lag in interferon production compared to adults, but the activated neonatal T lymphocytes are able to lyse infected cells when tested using a variety of cytolytic measurement assays (Yorty and Bonneau, 2004b; Zahwa et al., 2008).

C. Neuro-endocrine immune interaction

1. Introduction

Several regulatory processes exist within the immune system to promote self-monitoring and prevent uncontrollable immune responses (Syvalahti, 1987). There is an abundance of evidence suggesting that immune modulation is achieved through external regulation. Depending on the duration and intensity of stress, neuroendocrine signals have both synergistic and antagonistic effects on the immune system. Thus central nervous and endocrine systems interact in a bidirectional fashion with each being able to
up- or down-regulate the other based on the psychological and physical conditions (Antoni, 2003; Shanks et al., 1998). The continued ability to adapt to surrounding environmental variables increases chances for survival. In the psycho-neuro-endocrine interaction, the individual responds to psychological stimuli by activating various components of the neurological system that exerts synergistic and antagonistic effects on the immune system, ultimately improving its chances of survival.

Psychological stress is broadly defined as a perceived stimuli that alters the homeostatic state (Chrousos and Gold, 1992; Johnson et al., 1992; Sternberg et al., 1992b). The hallmark of a classical stress response (discussed in depth elsewhere in this literature review) involves activation of the hypothalamic-pituitary-adrenal (HPA) axis among other systems. Stress induces the release of corticotrophin-releasing hormone (CRH) by neurons in the hypothalamus, CRH acts on cells in the anterior pituitary gland resulting in the release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH acts on cells in the adrenal cortex resulting in increased synthesis and release of glucocorticoids (Biondi and Zannino, 1997). All nucleated cells, including lymphocytes, have intra-cytoplasmic corticosteroid-specific receptors allowing for immune regulation by glucocorticoids (Cupps and Fauci, 1982).

There is evidence suggesting that prenatal stress impacts the immune competence of neonates (Coe et al., 1999). Additional evidence suggests that the immunological consequences of stress are long lasting, even after the removal of the stressor (Benschop et al., 1994; Brosschot et al., 1994; Matalka, 2003). Human subjects who had an unpredictable emotional stress were monitored for up to six-months. Stressed subjects exhibited functional alterations in immune parameters up to forty days post-incident.
A low level of glucocorticoids is thought to be protective by providing energy substrates and modulating the immune system. During chronic stress, the level of glucocorticoids rises and remains elevated for a period of time after elimination of stress stimuli, extending its immune modulation effects (Chrousos and Gold, 1992; Johnson et al., 1992; Sternberg et al., 1992b). Prolonged immune modulation resulting from elevated glucocorticoids in response to prolonged or intermittent chronic stress, has been shown to be detrimental when coupled with HSV infection (Bauer et al., 2001; Nair and Bonneau, 2006). As mentioned earlier, neuroendocrine interaction is bidirectional. IL-1, a peripherally generated cytokine, is able to activate the HPA axis resulting in the down regulation of immune responses acting as a negative feedback signal (Sternberg et al., 1992a).

There are differences between species with regards to the maturation of the HPA axis relative to birth. For example, in animals that deliver precocious births (such as sheep guinea pigs, and primates) the brain and HPA axis are fully developed at the time of birth. On the other hand, in non-precocious births (such as rats, rabbits, and mice) the brain and the HPA axis development takes place during the first few weeks of the neonate’s life (Matthews, 2002). In fact, the HPA axis in the neonates of C57BL/6 mice is considered to be hyporesponsive to stress within the first ~11-14 days life, interestingly, this stress hyporesponsiveness is not observed when neonates were exposed to the same stressor at 21 days of age, as measured by the serum corticosterone level after the stress session (Yorty and Bonneau, 2004a).
2. Hypothalamic-pituitary-adrenal axis (HPA)

a. Introduction

Several neuropeptide-secreting systems are activated in the brain in response to stress. One of several goals of this orchestrated activation is the release of glucocorticoids from the cortex of adrenal glands. These secreted glucocorticoids bind nuclear receptors in almost all nucleated cells. These glucocorticoids provide a feedback mechanism to further regulate the production process (de Kloet et al., 2005; Schmidt et al., 2005). The effects of glucocorticoid have been described as a “binary master switch”, one that controls many neuronal and immune responses (de Kloet et al., 2005; Schmidt et al., 2005). Incoming sensory signals, informing the brain of stress, are continually assessed by the limbic brain structures (hippocampus, amygdale, and prefrontal cortex) to maintain homeostasis (de Kloet et al., 2005; Schmidt et al., 2003). This process of restoring homeostasis is termed the “stress response” (Selye, 1941a, c). The immediate firing mechanism to stressors is the activation of the sympathetic nervous system that leads to a sharp and rapid increase in serum concentration of adrenalin (from synapses) and nor-adrenalin (from adrenal medullary cells). This rapid firing mechanism is followed by another potent and “binary master switch” regulation of several neuronal and immune responses through the release of glucocorticoid approximately 15-30 minutes after the exposure to the stressor.
b. Neonates

As has been previously demonstrated (Angelogianni and Gianoulakis, 1989; Schmidt et al., 2003; Walker et al., 1986; Yorty and Bonneau, 2004a), neonatal mice less than approximately 14 days of age, do not exhibit a stress response as measured by activation of the HPA axis and the concomitant increase in corticosterone. However, it is important to note that unlike rodents, human neonates are able to elicit their own stress response as measured by increases in serum cortisol. Therefore, our studies in mice serve as a model to isolate the effects of both transmammary- and endogenously-acquired cortisol on neonatal immune function.

c. Experimental stress models

Over the years, several stress models have been used to study how hormones produced in response to stress mechanistically restores homeostasis. Humans were subjected to laboratory-induced stressors including, but not limited to, speech-stress test and arithmetic-stress tests (Burleson et al., 2003). Additionally, life stressors such as the care for an elderly relative or the death of a loved one have also been examined for their effects on individuals experiencing such events. Medical students, West Point cadets, caregivers for family members, cancer patients, or post-operative patients were studied over the years to better understand the stress response (Webster Marketon and Glaser, 2008). Laboratory animals have also been used to further elucidate the effects of stress. To mechanistically study the effects of stress, several research groups have used models
such as rational stress, footshock, restraint stress, and social disruption stressor. Both human and animal stress models shared one common finding: regardless of the stress used, elevated serum glucocorticoid levels are observed (Bonneau et al., 1993c; Hu et al., 2003; McCarty et al., 1991; Padgett et al., 1998; Shanks et al., 1990; Shanks et al., 1991; Webster Morakton and Glaser, 2008).

It has been clearly demonstrated that psychological stress activates that HPA axis resulting in elevated glucocorticoids in the circulation (Balcombe et al., 2004; Leo et al., 1998; Nair and Bonneau, 2006; Sheridan et al., 1991; Yorty et al., 2004). A number of studies in humans have described a variety of psychological stressors often experienced during the prenatal (Coussons-Read et al., 2007; Coussons-Read et al., 2005) or postpartum period (Gröer et al., 2005; Gröer et al., 2002). For example, postpartum fatigue, disturbed sleep, emotional tension, and the daily lifestyle changes often experienced by new mothers are associated with a higher level of stress and, subsequently, elevated serum glucocorticoid levels (Gröer et al., 2005; Gröer et al., 2002). Pre-term delivery has also been shown to be associated with increased levels of glucocorticoids in the milk (Gröer et al., 1994) and may be a function of the variety of stressors that are often associated with this type of delivery. This latter finding is particularly significant given that mothers are able to transfer this, and other hormones that reflect their physiological state, to their offspring via milk (Koldovsky, 1980; Kulski and Hartmann, 1981; Rosner et al., 1976; Tucker and Schwalm, 1977; Yeh, 1984). To determine the impact of stress-associated levels of maternal corticosterone on the neonatal adaptive immune response, we utilized an approach to increase corticosterone by providing corticosterone-supplemented drinking water to lactating mice (Catalani et
al., 2000; Yorty et al., 2004). This approach mimics chronic stress-induced levels of corticosterone but avoids the long-term separation of neonates from their mothers that would be inherent to the use other murine models of stress, such as restraint.

d. HPA activation in response to stress

The classic model for glucocorticoid action through its interaction with the glucocorticoid receptor and the formation of glucocorticoid responsive element (GRE) and glucocorticoid responsive unit (GRU) does not fully explain some of the rapid effects of glucocorticoids, thus, leading to the assumption that non-genomic actions are mediated by distinct membrane receptors (Chen and Qiu, 1999; Norman et al., 2004). Recent evidence described distinctive hormone-binding cellular receptor in mammalian cells linked to intracellular pathways acting through G-protein-coupled receptors and kinase pathways (Chen and Qiu, 1999; Evans et al., 2000; Norman et al., 2004; Powell et al., 1999).

e. Glucocorticoid receptor antagonist mechanism of action

Mifepristone, RU486, does not block the synthesis of glucocorticoid or progesterone, but rather competes for binding with these two hormones for glucocorticoid and progesterone receptor, respectively. In vivo, low doses of RU486 bind to the progesterone receptor with high affinity. A larger dose of RU486 is required for anti-glucocorticoid effects. The anti-glucocorticoid mechanisms are not without
complications. For example, in Cushing’s disease patients with prolonged exposure to elevated levels of cortisol not associated with HPA axis over-activation, the use of RU486 has proven beneficial in suppressing the deleterious effects of hypercortisolism (Johanssen and Allolio, 2007). On the other hand, this is not the case when HPA axis impairment is the cause of hypercortisolism. The administration of RU486, in the HPA axis impairment cases, interferes with the glucocorticoid negative-feedback mechanism ultimately resulting in further increased release of ACTH with subsequent increased circulating glucocorticoids (Baulieu, 1991a, b).

D. Neonatal immune system

1. Dependence on maternal interaction in utero

The intimate interaction between the mother and infant has both physical and psychological life-long consequences (Amoudruz et al., 2005; Catalani et al., 2000). This interaction begins in utero and extends postnatally for several years. During pregnancy, nutrients along with hormones are passively provided to the fetus. It has been documented that prenatal stress is associated with HPA axis alterations in many species, including humans and mice (Welberg and Seckl, 2001). However, no cross-species general statement can be made regarding prenatal maternal influence on the neonatal HPA axis and immunocompetence (Shanks and Lightman, 2001). What can be said is that maternal HPA axis activation exerts long-term effects on neonatal
immunocompetence and its HPA axis reactivity (Shanks and Lightman, 2001). These effects could be implicated in susceptibility to infection and autoimmune disease. However, HPA axis plasticity compensates for in utero effects evident by the absence of differences between offspring born to stressed or non-stressed mothers when they are cross-fostered by non-stressed mothers (Shanks and Lightman, 2001). The reverse also holds true; that is, cross-fostering offspring born to non-stressed mothers to stressed mothers results in neonatal immunosuppression (Shanks and Lightman, 2001). Together, these findings imply that maternal stress is mediated prenatally via the placenta and postnataally via the mothers behavior and/or milk-borne factors (Shanks and Lightman, 2001).

Prenatal stress exerts effects on both the innate and adaptive immune systems. For example, prenatal stress reduces the cytotoxicity of NK cells from the spleens of prenatally stressed rat pups in response to mitogen (Klein and Rager, 1995). Consistent with rodent data, prenatally stressed primates also exhibited altered antibody levels and reduced NK cytotoxicity (Shanks and Lightman, 2001).

2. Impact of early experiences on the neonatal immune system development

The interaction between the mother and the infant extends for several weeks, and sometimes months to years depending on the species. This interaction is not only physical by also psychological. At birth, the neonate’s immune system is considered naive due to lack of exposure to foreign antigens. Therefore, its ability to respond
efficiently to infection is, as discussed in other sections of this review. It is during this period that neonates are exposed to dietary pathogens that they need to develop tolerance against. A strong immune response against dietary pathogens could be deleterious. The delicate balance between tolerance and efficient immune education is achieved via interaction with maternal factors provided through the milk and through the maturation of the neonate’s own immune system. Factors supplied through the mother’s colostrum and milk can be grouped into four main categories comprising maturational, immunomodulatory, anti-inflammatory, and anti-microbial (Kelly and Coutts, 2000). Of interest to this review are the last three categories, while it suffices to say that colostrum and milk have been shown to promote the maturation of the infant’s intestinal epithelium (Kelly and Coutts, 2000).

Maternal milk plays an important role in immuno-modulation of the newborn’s immune system. Specifically, comparing the ability of breast-fed to artificially-fed neonates to mount an antibody response against vaccines demonstrates that breast-fed neonates exhibit an enhanced antibody titer to some but not all vaccines (Pickering et al., 1998). Similar studies examining the cell-mediated immune response in breast-fed and artificially-fed neonates demonstrated that, following exposure to maternal milk, neonates exhibited a decrease in the CD4:CD8 cell ratio, an increase in IFN-γ, and an increase in the number of NK cells (Hawkes et al., 1999; Pabst et al., 1997). In addition to the modulation effects described, breastfeeding also has immunosuppressive effects. These two findings are not contradictory. The immune modulation is necessary for the development of an effective immune system, while the immune suppression is needed to develop tolerance to dietary and harmless antigens (Kelly and Coutts, 2000). Breast milk
contains IgA antibodies and IL-10, both of which exert immunosuppressive effects on the neonate’s immune system (Garofalo et al., 1995). These findings indicate the cytokine milieu in the neonate is influenced by maternal factors. In humans, maternal T cells actively home to the mammary gland and are transferred to the fetus via the milk. These cells are CD45RO+ (memory phenotype); (Bertotto et al., 1990). These cells are believed to either remain locally in the newborn’s intestinal lymphoid tissue or migrate into the circulation (Goldman and Goldblum, 1997; Xanthou, 1997). Additionally, these cells express very high levels of CD40L believed to compensate for the immature neonatal immune system providing strong signals leading to neonatal T cell activation (Bertotto et al., 1990). From the immunomodulatory prospective, breast milk alters the cytokine milieu against which an immune response is elicited in neonates resulting in both adequate response to harmful pathogens and tolerance to dietary antigens and harmless commensal bacteria.

Breast milk plays an anti-inflammatory role during the neonate’s developmental stage. IL-10 and transformation growth factor-β (TGF-β) have also been documented to be present in breast milk (Garofalo et al., 1995; Letterio et al., 1994). These two cytokines are known to have anti-inflammatory activities. Studies performed using TGF-β knockout mice, in which gene disruption causes diffused and lethal inflammation, demonstrated that exogenous TGF-β was sufficient to quell the infection and reduce mortality (Letterio et al., 1994). In the same study, maternal lymphocytes and macrophages present in the breast milk contained TGF-β and this cytokine was taken up rapidly by the neonate’s intestine (Letterio et al., 1994). IL-10 on the other hand has
been shown to inhibit the production of pro-inflammatory cytokines, limit the Th1 response and work synergistically with TGF-β (Kelly and Coutts, 2000).

Breast milk also serves as an anti-microbial agent. It has been widely documented the IgA antibodies are present in abundance in breast milk, at an even higher concentrations than in serum (Kelly and Coutts, 2000; Yorty and Bonneau, 2003, 2004b). This antibody plays an important role in pathogen clearance as it prevents the attachment of pathogens to the intestinal wall by competitively binding to bacterial antigens (Schroten et al., 1998). Other factors in the milk that play an inflammatory role have been described. Of interest are oligosaccharides. These compounds are present in abundance in breast milk and share homology with cell surface pathogen receptors, making them perfect candidates to compete with true pathogens for cell adhesion and entry resulting in preventing pathogen invasion (Newburg, 1999). Maternal milk contains a wide range of factors that neutralize the pathogen and/or prevents attachment and entry with the ultimate goal being protection of the neonate.

3. Maternal influence on neonatal immune system

a. Introduction

The continuous interaction between the mother and her neonate starts at conceptions and extends beyond delivery. This interaction extends for many weeks, if not years, into the life of the child. Maternal immune status developed throughout the
mother’s life have been shown to be capable of modulating the neonate’s immune response (Fagoaga and Nehlsen-Cannarella, 2002). Evidence suggests that this modulation not only impacts humoral immune but also extends to cell-mediated immunity (Cramer et al., 1974; Field and Caspary, 1971; Russell, 1975).

b. Prenatal

Transplacental transfer of a wide range of antibodies, and possibly antigens, occurs during the gestational period. Utilizing Fcγ receptor (Brambell receptor) for transplacental transfer, it has been documented that at the time of delivery a full-term human neonate’s serum IgG level is about 90% of levels of its mother’s serum IgG (Hanson et al., 2003; Junghans, 1997). However, this process is not random as some maternal IgG antibodies with certain specificities and high avidity are better at transplacental transfer than others (Avanzini et al., 1998). These antibodies provide protection to the neonate during the first few months of life, sparing the neonate the energy consuming process involved in generating an immune response to fight the great number of pathogens (some of which are normal gut flora) they will encounter (Hanson et al., 2003).

c. Postnatal

Maternal care for the neonate does not simply end with the act of delivery. Breastfeeding following delivery lasts in some cultures for up to 24 months. One cannot
underestimate the value of breastfeeding. The benefits extend into adulthood, and possibly for the individual’s entire life (Hanson, 1998). On ultrasound examination, the thymi of neonates that were breastfed were twice the size of their counterparts who were not breastfed (Hasselbalch et al., 1996). Additionally, the outcome of mother-to-sibling kidney transplantation is much better if the sibling was breastfed as a neonate, suggesting that major histocompatibility tolerization occurs early in life for these neonates (Campbell et al., 1984; Kois et al., 1984). Even though human neonates are totally dependent on their mother’s breast milk for nutrition in the first few weeks of life, only 30% of the proteins contained in this milk are for the sole purpose of host defense (Hanson et al., 2003). As discussed elsewhere in this literature review, the most predominant isotype in the milk is secretory IgA (SIgA) with concentrations ranging from 0.5 to 1.5 g/L. The concentrations of other isotypes such as IgG and IgM are small compared to SIgA (Hanson, 1998; Hanson et al., 2003). This SIgA is produced by cells that have been exposed to the mother’s lymphocytes that have been activated in the Peyer’s patches. Thus, it provides protection against all pathogens the mother has, or that were previously present, in her gut (Hanson et al., 2003). When the neonate is colonized with gut flora postnatally, a massive energy consuming immune response is averted by the presence of these SIgA antibodies that were transferred from mother to neonate via the milk (Hanson et al., 2003). The mechanism of action for SIgA involves preventing colonizing pathogens from adhering to mucosal surfaces, thereby averting the inflammatory process.

Another major protein that is transferred from mother to neonate via the milk is lactoferrin (LF). About 1-4 g/L of LF is transferred in the milk, and like SIgA this
protein is relatively resistant to enzymatic degradation (Hanson et al., 2003). LF plays an important role in host defense against colonizing pathogens. Specifically, LF acts as a microbicidal, immunostimulatory, and anti-inflammatory agent by downregulating several pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL8 (Baveye et al., 2000; Hanson, 1998; Hanson et al., 2003). Intriguingly, the antibody and T cell response to vaccination after breastfeeding is markedly better than in non-breastfed counterparts (Pabst et al., 1997).

E. The impact of stress on lactation

1. Introduction

Since ancient times, scientist have been mesmerized by the origin of milk. Two anecdotal observations led to the formation of the first theory on milk formation. The first observation was the absence of flower’s during pregnancy; and the second being the “tingle” women felt in their lower abdomen during breastfeeding. The “scientific” description for these two observations is the absence of menstruation during pregnancy and the uterine contractions associated with oxytocin release, respectively. Hippocrates (460-375 BCE) believed, and was later supported by Claudius Galen (129-199 CE), that there was a vessel connecting the uterus to the breast. This vessel supposedly enabled the menstrual blood that “nourished” the fetus to continue doing so through breastfeeding. These observations went mostly unchallenged even by Leonardo Da Vinci (1452-1519),
who examined Galen’s work closely and perpetuated many of his anatomical errors. Da Vinci even incorporated Galen’s belief in the “vas menstrualis” in his 1492 drawing “Coition of a Hemisected Man and Woman” (Figure 1). Gasparo Aselli (1627) perforated a white cord that drained a dog’s intestine and noted that a “milk-like fluid gushed out”. Based on this observation, he postulated that milk was derived from chyle (a substance formed during the digestion of fatty foods and is drained by the lymphatic system) and is delivered to the breast via “absorbents” (lymphatic vessels). It wasn’t until 1840 when Astley Cooper, a scientist who did not report anything he did not observe himself, criticized Aselli’s chyle theory by stating: “A most extraordinary opinion [Aselli’s chyle theory] has been broached, an opinion at variance with the nature of the fluid, entirely inconsistent with every injection which I have made, as they all pass from, and not towards, the breast, and irreconcilable with the valvular structure of the vessels”. Even 100 years later, the claim still persisted that milk was made during the milking process, an observation based on dairy cows. This is quite a fascinating assumption since almost 500 mL of milk can be excreted from a cow in 10 seconds. This brought about the distinction between milk secretion and milk ejection, a distinction made by Gaines in 1915. He postulated that the former was continuous and the latter discontinuous (Gaines, 1915). He further postulated that the reflux arc involving the central nervous system is required for milk release as an anesthetized animal failed to yield sufficient milk to suckling pups (Gaines, 1915). Another four decades passed before another major breakthrough in lactogenesis occurred. In 1942, Mavis Gunther described, in the Canadian Medical Association Journal, the first correct physiological description of milk ejection (Gunther, 1942). Her findings were further confirmed when
Oxytocin was used to reverse a decreased milk release reflux (Newton and Newton, 1948). Given the nutritional dependency of neonates on their mothers for the first few weeks of life, milk synthesis and excretion is resistant to many environmental factors (Whitehead et al., 1986). Despite an abundance of scientific and anecdotal evidence regarding milk synthesis and excretion, some of the traditional thoughts still exist today in health professional circles.

**Figure 1.**

A cutout from Leonardo Da Vinci’s 1492 drawing title “Coition of a Hemisected Man and Woman”. The arrow points to Da Vinci’s depiction of the “vas menstrualis”, a connection between the uterus and the breast. This drawing is a culmination of the prevailing view on the origin of milk at the time.
2. Nursing and dampened stress response

It is absolutely critical for neonates to obtain sufficient quantities of quality nourishment. In humans, and many other species, the total dependence of neonates on their mothers during the first few weeks of life makes maternal milk one, if not the only, source of nutrition for neonates. What distinguishes human neonates from other neonates is that they are born with sufficient energy storage. This storage is useful since breastfeeding in the first 24-48 hours yields a highly concentrated solution termed “colostrum”. In humans, this colostrum is secreted in small quantities (~30 mL/24 h). As counterintuitive as this sounds, it is actually for the benefit of the neonate. This colostrum delivers potent native immune components such as lactoferrin and SIgA. These components help providing adequate surface protection for the gastrointestinal (before the introduction of large quantities of milk) and respiratory systems (Saint et al., 1984).

It is generally believed that mammary glands are fully developed and are ready to produce and eject milk by the time the mother delivers her infant (Neifert et al., 1985). However, it is also believed that physical and psychological factors contribute to milk production and the excretion process. Although oxytocin and glucocorticoids are among the primary lactogenic hormones, others also have been implicated in this process (Lau, 2001; Neville, 2001; Neville and Morton, 2001). When lactation failure cannot be explained by mammary gland insufficiency, it is usually blamed on stress. However, different stressors have varying effects on lactation (Lau, 2001). One fact remains; the role of glucocorticoid in lactogenesis is not clearly understood.
Although it is generally believed that, in the absence of mammary gland defects, failure to lactate is due to stress, it is not clearly understood how neuroendocrine hormones generated during the stress response affect milk production and/or excretion. The notion that stress negatively impacts lactation is based on animal studies where lactation is decreased in response to a variety of stressors (Lau, 2001). Despite this notion, there is a lack of studies describing the effects of stress on lactation in humans. Given the complexity of hormones involved in milk synthesis and ejections, a variety of stressors could have a wide range of effects on synthesis and ejection (Lau, 2001). It is clear that milk ejection is dependent on the availability of milk (milk secretion). Stressors that interfere with milk synthesis will eventually result in reduced milk ejection (Lau, 2001). Additionally, “breast emptying” also affects milk synthesis. Consequently, stressors that interfere with milk ejection will eventually lead to decreased milk synthesis.

Studies have also confirmed that in many species, lactation dampens the hypothalamic-adrenal response (Altemus et al., 2001). When lactating and non-lactating female non-human primates were exposed to the same stressor, lactating females exhibited higher levels of serum cortisol, suggesting that nursing per se did not attenuate the HPA axis as documented in other species (Maestripieri et al., 2008). However, when ewes were subjected to restraint stress to activate their HPA axis, as measured by circulating ACTH concentration, the stress response was more prominent in non-lactating females than lactating ones (Tilbrook et al., 2006). This “HPA attenuation” is thought to be mediated by oxytocin which reduces CRF release resulting in decreased ACTH production, ultimately culminating in reduced circulatory glucocorticoid levels (Boutet et al., 2006; Lightman and Young, 1989). Oxytocin is known to stimulate maternal
interaction and attachment between the mother and offspring (behavioral aspects). Additionally, oxytocin reduces serum cortisol levels and blood pressure (Uvnas-Moberg et al., 2001). The act of suckling itself is also known to induce “HPA attenuation” through the increased serum levels of oxytocin and somatostatin resulting in decreased cortisol levels in cows (Lupoli et al., 2001). Studies in rats where prolonged exposure to stress in the form of separation or introduction of an intruder male, resulted in reduced milk release in nursing females (Lau and Simpson, 2004).

Dairy cows are best model animal for milk synthesis and excretion. In this model, the administration of cortisol resulted in serum levels significantly higher than baseline. More importantly, this administration of cortisol, whether in a single dose or two doses per day, did not affect milk yield from these cows (Mayer and Lefcourt, 1987). One concludes from these studies that “HPA attenuation” and reduced milk yield (synthesis and/or excretion) varies from one species to another, and is dependent on the form and duration of stressor.

F. Summary

In summary, there is an abundance of evidence that describes the ability of stress to activate the HPA axis culminating in the production of several “stress hormones” that include, but are not limited to glucocorticoids (cortisol in humans and corticosterone in rodents). These glucocorticoids bind their natural receptor, GR, in the cytoplasm of all nucleated cells which then dimerizes and translocates to the nucleus where it binds the GRE altering the binding sites for several transcription factors in target cells. The
ultimate goal of this network is restoring homeostasis that was originally disrupted by the stressful event. Mammalian neonates are almost entirely dependent on their mothers not only for nutrition but also for general care during the first few weeks of life. However, this dependence exposes the neonate to a variety of factors that are transmitted in the mother’s milk. Some are of great benefit to the neonates such as SIgA, LF, and nutrients that the neonate desperately needs for survival. Other factors, such as stress hormones reflecting the mother’s psychological and hormonal status, are transmitted to the neonate possibly producing a wide range of effects on the neonate, a hypothesis that is tested herein.

Interestingly, the complex HPA network is activated in response to physical and/or psychological stress enabling us to examine the effects of stress on the cell mediated immune response in neonates that were exposed to maternally-derived corticosterone. Our findings are significant in that they emphasize the potential impact of corticosterone on a neonate’s ability to generate protective immunity not only following natural exposure to pathogens but also in response to the many vaccinations that neonates typically receive.
Chapter II: Elevated Maternal Corticosterone During Lactation Hinders The Neonatal Adaptive Immune Response to Herpes Simplex Virus (HSV) Infection

A. Abstract

Most neonatal mammals depend almost entirely on their mother’s milk for immune-mediated defenses against infectious pathogens to which they may be exposed during the first few weeks of life. During this time, the immune system of these neonates is considered to be relatively immature and reliant primarily on maternally-derived antibodies. However, neonates soon acquire the ability to generate adaptive immune responses to some of these pathogens. In adults, products of the nervous and endocrine systems that are elicited by psychological stress are known to modulate a variety of immune responses. Psychological stressors are well recognized for their ability to increase corticosterone levels. We have previously used a murine model to show that stress-induced increases in maternal corticosterone result in the transfer of increased levels of corticosterone to neonates via the mother’s milk. However, little had been known about the impact of this increase on the neonatal cell-mediated immune response against viral pathogens. The studies described herein examined the effects of increases in maternally-derived corticosterone on the neonatal cell-mediated immune response to HSV infection and its associated neonatal morbidity and mortality. Drinking water containing corticosterone was made available to nursing mothers for a period of 6 consecutive days beginning on either the day of delivery or 6 days post-delivery. At 12 days of age, all neonates were infected with HSV-1 in the rear footpads and the HSV-
specific CTL response was determined in the draining popliteal lymph nodes five days post-infection. Only those neonates that were nursed by mothers whose water contained corticosterone exhibited a decrease in the proliferative ability of splenic-derived cells due to the reduction of IL-2 production and IL-2Rα expression by these cells. These neonates also exhibited a decrease in the number and function of HSV-1 gB\textsubscript{498-505} peptide-specific CD8\textsuperscript{+} T cells as measured by tetramer analysis, CTL lytic activity, expression of CD107a, cytokine production, and proliferation. Additionally, these HSV-infected neonates exhibited increases in the incidence of both hind-limb paralysis and mortality. Together, these studies indicate that exposure of neonates to maternally-derived corticosterone via the milk hinders their ability to generate an adaptive cell mediated-immune response to a viral infection and illustrate the potential importance of maternal stress on neonatal resistance to infectious pathogens.
B. Introduction

Psychological stress activates the HPA axis resulting in an elevated synthesis and release of immunomodulatory hormones such as glucocorticoids from the adrenal glands (de Kloet et al., 2006; Kudielka and Kirschbaum, 2005; Selye, 1941b). A number of studies in humans have described a variety of psychological stressors that are often experienced during the prenatal (Coussons-Read et al., 2007; Coussons-Read et al., 2005) or postpartum period (Gröer et al., 2005; Gröer et al., 2002). For example, the postpartum fatigue, disturbed sleep, emotional tension, and the daily lifestyle changes that are often experienced by many new mothers are associated with a higher level of stress and elevated serum cortisol levels (Gröer et al., 2005; Gröer et al., 2002). Pre-term delivery has been shown to be associated with increased levels of cortisol in the milk (Gröer et al., 1994) and may be a function of the variety of stressors that are often associated with this type of delivery. This latter finding is particularly significant given that mothers are able to transfer this and other hormones that reflect their physiological state to their offspring via milk (Koldovsky, 1980; Kulski and Hartmann, 1981; Rosner et al., 1976; Tucker and Schwalm, 1977; Yeh, 1984). Even though this transfer of hormones has been well recognized for at least thirty years, the impact of this transfer on the neonatal immune response to infectious pathogens has yet to be determined.

Neonatal mammals depend almost entirely on their mother’s milk for immunologic protection (Newburg, 2005). It is through this milk that the mother passively provides her neonates with antibodies against a variety of infectious pathogens. These antibodies serve to not only complement the developing immune system of the
neonate, but to also actively ‘educate’ the neonatal immune system on how to generate a proper adaptive immune response against antigenic challenges (Kelly and Coutts, 2000). Studies from our laboratory have demonstrated that the transfer HSV-specific antibody protects newborn mice against HSV infection (Yorty and Bonneau, 2003, 2004a, b; Yorty et al., 2004). However, for those pathogens to which the mother has not previously been exposed, the need for the neonate to develop its own adaptive immune responses following exposure to infectious pathogens is absolutely critical. Despite the clear existence of stress-neuroendocrine-immune interactions in adults, there is a dearth of knowledge as to the role that neonatal exposure to corticosterone plays in the development and function of adaptive immunity during the initial encounter with a pathogen and its effect on neonatal susceptibility to infection. Studies in our laboratory have demonstrated that acute maternal stress elevates corticosterone levels in maternal serum and milk. This corticosterone-containing milk is passed to the nursing neonates resulting in the elevation of their own serum corticosterone levels (Yorty and Bonneau, 2003). We have also shown that elevated levels of corticosterone in the mother’s milk correlate with the increased HSV-associated mortality in neonates (Yorty and Bonneau, 2004a). To determine the impact of stress-associated levels of maternal corticosterone on the neonatal adaptive immune response, we utilized an approach to increase corticosterone by providing corticosterone-supplemented drinking water to lactating mice (Yorty et al., 2004). This approach mimics chronic stress-induced levels of corticosterone but avoids the long-term separation of neonates from their mothers that would be inherent to the use other murine models of stress, such as restraint. The ability of a neonate to develop adaptive antiviral immune defense mechanisms is potentially
regulated by stress-associated, neuroendocrine-derived peptides and hormones — a hypothesis that is tested in the studies described herein.

Several studies from our laboratory have described the impact of stress and elevated corticosterone on the adult CTL response against HSV infection (Anglen et al., 2003; Bonneau, 1996; Bonneau et al., 1990; Bonneau et al., 1991b; Leo and Bonneau, 2000a, b; Leo et al., 1998; Nair and Bonneau, 2006; Nair et al., 2007a). However, until now, the effects of maternal stress, and the subsequent elevation of maternal and neonatal serum corticosterone, on the neonatal antigen-specific cellular immune responses have not been described. The studies described herein utilized HSV-1 as a model pathogen to determine the impact of maternally-derived corticosterone on the quantity and functions of HSV-specific CD8⁺ T cells and, in particular, for those T cells that are specific for the H-2Kᵇ-restricted, HSV-1 gB₄₉₈-₅₀₅ epitope. This epitope has been identified as the immunodominant CTL-recognition epitope in the immune response to HSV-1 infection in C57BL/6 mice (Bonneau et al., 1993a; Bonneau et al., 1993b; Fu et al., 1996; Salvucci et al., 1995). Using well-established murine models of neonatal HSV infection (Bonneau, 1996; Bonneau et al., 1990; Wiley et al., 2001; Yorty et al., 2004) and state-of-the-art measures of antiviral immunity, we demonstrated that neonatal exposure to maternally-derived corticosterone increases neonatal morbidity and mortality associated with HSV infection and suppresses the number and function of HSV-specific CTL.
C. Materials and Methods

1. Animals and experimental design

Four to six week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a controlled-temperature room (22-25°C) with a 12:12 hour light/dark cycle (lights on 0700-1900) and were allowed one week to acclimate to these conditions before any experimental manipulations were performed. Standard rodent diet (Harlan Teklad, Cat. No. 2018) and water were made available ad libitum. At the end of the acclimation period, individual female mice were housed with one male C57BL/6 mouse. The breeding pairs were left undisturbed for 14 days, at which time the males were removed and females were housed one mouse per cage. Litters were typically comprised of five to eight neonates; litters with less than five neonates were augmented to seven neonates per mother, and litters of greater than seven were culled to seven neonates per mother on the day of birth. For each experiment presented herein, at least six litters were designated for each experimental manipulation (Corticosterone, Vehicle …etc). Popliteal lymph nodes were pooled from three siblings per litter. Spleen samples were not pooled. Data shown represents experiments performed on the same cells, i.e. if pooled cells were used they were divided equally among the experiments. All mice were handled in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and the National Institutes of Health.
2. **Cell lines and media**

The B6/WT-3 cell line (Pretell et al., 1979) was maintained in Dulbecco's-modified Eagle's minimal essential medium (DMEM), (Gibco; Carlsbad, CA) supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS), 20 mM Hepes buffer, and 0.075% (wt/vol) NaHCO₃. Lymphocyte cultures utilized Iscove's-modified Dulbecco's medium (IMDM); (Gibco) supplemented with 10% FBS (vol/vol), 0.225% (wt/vol) NaHCO₃, 25 mM Hepes buffer, and 20 mM 2-mercaptoethanol. Vero cells were grown in media 199 (Gibco) containing 4% FBS, 4% newborn bovine serum (NBS), 8% tryptose phosphate broth, and 0.225% (open vessel) or 0.075% (closed vessel) NaHCO₃. All media also contained 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate.

3. **Virus**

HSV-1 strain Patton virus stocks were prepared in Vero cells by infection at a multiplicity of infection (MOI) of 0.01 and virus titers were determined by plaque assay on Vero cells. Virus stocks were stored at -70°C.

4. **Synthetic peptides**

Synthetic peptides corresponding to the HSV-1 CTL recognition epitope gB₄₉₈–₅₀₅ (SSIEFARL) (Bonneau et al., 1993c) and ovalbumin amino acid residues 257-264
(OVA_{257-264}; \text{SIINFEKL}) were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050) MilliGen PepSynthesizer. Peptide stock solutions were prepared by solubilizing the lyophilized peptides in dimethyl sulfoxide (DMSO) and adjusting the concentration to 1 mM with non-supplemented RPMI 1640 medium.

5. **Exogenous corticosterone administration**

Corticosterone was dissolved in 30% (wt/vol) 2-hydroxypropyl-β-cyclodextrin (HBC Cat. No. H107-100G; Sigma-Aldrich, St. Louis, MO) at 37°C with repeated vortexing and diluted to 200 µg/ml in tap water. Female mice were provided water containing either 200 µg/ml corticosterone (MP Biomedicals, Cat. No. 101416; Solon, OH) or as a control, vehicle (0.6% HBC); (Dhabhar and McEwen, 1999), ad libitum beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for six consecutive days. Water was changed every other day. To minimize the potential impact of animal handling on immunological measures, the bedding in all cages was changed at fourteen days post-delivery (Balcombe et al., 2004). When indicated, neonates were weaned by gender at 30 days of age.

6. **Serum and milk collection**

Adult female mice were rapidly euthanized via cervical dislocation and cardiac puncture was performed to obtain blood samples. Neonatal mice were anesthetized in a
saturated atmosphere of isoflurane, decapitated, and trunk blood was collected. Milk curd was collected from the neonate’s stomach via dissection. These milk samples were diluted 1:1 (based on weight), with supplemented DMEM. This diluted milk was sonicated for 2 minutes using a 40 watt sonicator and centrifuged for 10 minutes at 16,000 x g. The supernatant was collected from below the lipid layer and stored at -70°C. All blood samples were centrifuged at 16,000 x g for 2 minutes and serum was collected and stored at -70°C.

7. **Quantification of corticosterone**

The levels of corticosterone in both serum and milk were determined using a radioimmunoassay-based (RIA) kit (MP Biomedical, Cat. No. 07-120103). These levels were calculated using a standard curve generated from standards containing 0-1000 ng/mL of corticosterone.

8. **Generation of HSV-specific immune cells with cytolytic activity and IFN-γ secreting capabilities**

At 12 days of age, mice were infected in the rear footpads with 1 x 10^6 PFU of HSV-1 Patton in a volume of 0.01 mL and were euthanized five days post-HSV-1 infection via cervical dislocation. Popliteal lymph nodes were removed, placed in supplemented IMDM, and mechanically dissociated by passage through a 60-gauge stainless steel mesh screen. Cells were then cultured at 4 x 10^6 cells/mL without
exogenous antigenic stimulation in supplemented IMDM for 3 days at 37°C in 5% CO₂. After 3 days in culture, the cells were pelleted by centrifugation at 60 x g for 5 min at room temperature. The supernatant was collected to determine the amount of interferon gamma (IFN-γ) and interleukin-2 (IL-2) synthesized by the cells in those cultures. The pelleted cells were washed three times and resuspended in supplemented IMDM for further analysis.

9. **gB<sub>498-505</sub> tetramer staining**

For the detection and quantification of gB<sub>498-505</sub> epitope-specific CD8<sup>+</sup> T lymphocytes, cells were first washed twice with FACS buffer, and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86; San Diego, CA) for 20 minutes on ice. Cells were then incubated for 30 minutes on ice with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat No. 551162) and a PE-labeled tetramer. This tetramer, which binds to the H-2K<sup>b</sup>-restricted, gB<sub>498-505</sub> T cell receptor complex (Blaney et al., 1998), was prepared as described by Altman et al. (Altman et al., 1996) and kindly provided by Dr. Satvir Tevethia (The Pennsylvania State University College of Medicine). To control for non-specific tetramer binding, a tetramer specific for the H-2K<sup>b</sup>-restricted influenza epitope NP<sub>366-374</sub> (kindly provided by Dr. Satvir Tevethia) was used. Following three washes with FACS buffer (PBS supplemented with 1% FBS and 0.02% [wt/vol] sodium azide), gB<sub>498-505</sub> epitope-specific CD8<sup>+</sup> T lymphocytes were quantified by flow cytometric analysis.
10. Early and late apoptotic cell staining

For the determination of early and late apoptotic cells, mice were euthanized via cervical dislocation five days post-HSV-1 infection. Popliteal lymph nodes were removed, placed in supplemented IMDM, and mechanically dissociated by passage through a 60-gauge stainless steel mesh screen. Cells were then suspended at 3 x 10^5 cells/mL in supplemented IMDM, cells were washed twice with FACS buffer, and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were then incubated on ice for 30 minutes with APC-conjugated anti-CD8 (eBioscience, Cat. No. 17-0081-83) antibodies and PE-conjugated gB_{498-505} tetramer. To control for non-specific tetramer binding, a tetramer specific for the H-2K^b-restricted influenza epitope NP_{366-374} was used. At the end of this staining period, cells were washed three times with FACS buffer and resuspended in 100 µL of Annexin V binding buffer (BD Biosciences Pharmingen, San Diego, CA, Cat. No. 51-66121E). 5 µL of Annexin V-FITC (BD Biosciences Pharmingen, Cat. No. 51-65874X) and 10 µL of 7-amino-actinomycin D (7AAD) (BD Biosciences Pharmingen, Cat. No. 559925) were added to each sample and the cells were incubated in the dark for 15 minutes at room temperature. At the end of this incubation period, 400 µL of binding buffer was added to each sample. The samples were analyzed by flow cytometry within one hour.
11. Splenocyte lymphoproliferation

To assess the lymphoproliferative ability of splenic-derived lymphocytes, mice were euthanized by cervical dislocation five days post-HSV-1 infection. Spleens were removed, placed in supplemented IMDM (Gibco), and mechanically dissociated by passage through a 60-gauge stainless steel mesh screen. Erythrocytes were lysed using hemolysis buffer (17 mM Tris; 0.14 mM NH₄Cl; pH=7.65) at 37°C for 5 minutes. The cells were then washed with supplemented IMDM, resuspended at 2 x 10⁶ cells/mL in Hanks' Balanced Salt with 0.01% (wt/vol) Bovine Serum Albumin (HBSS-BSA), and stained in the dark at room temperature for 10 minutes with 5 µM carboxy-fluorescein diacetate succinimidyl ester (CFSE). The cells were then washed three times with supplemented IMDM, and 5 x 10⁶ cells in a volume of 50 µL were placed in a 96-well round-bottom microtiter plates (Corning Inc., Corning, New York). To determine the T cell receptor (TCR)-dependent polyclonal mitogenic response, cells were cultured with 2.5 µg/mL of concanavalin A (Con A; Sigma-Aldrich). To determine the TCR-independent mitogenic response, cells were cultured with 2.5 µg/mL phytohemagglutinin M (PHA-M; Sigma-Aldrich). Other cells were cultured with supplemented IMDM and served as a non-stimulated control. All cells were incubated for three days at 37°C in 5% CO₂. At the end of this incubation period, cells were washed twice with FACS buffer, and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were then incubated for 30 minutes on ice with PE-labeled anti-CD8 antibodies (eBioscience, Cat.
No. 12-0081-82). The extent of lymphocyte proliferation profile was determined by flow cytometric analysis.

12. **IL-2 receptor staining**

For the detection and quantification of IL-2 receptor alpha subunit (IL-2-Rα) on CD8\(^+\) T lymphocytes, cells were washed twice with FACS buffer, and the CD16/CD32 Fc\(\gamma\) receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were then incubated for 30 minutes on ice with anti-CD8 APC antibody (eBioscience, Cat. No. 17-0081-083) and a PE-Cy7-conjugated anti-CD25 (IL-2Rα) antibodies (eBioscience, Cat. No. 25-0251-82). This antibody binds to the 55kDa IL-2 receptor α chain which is an integral part of the IL-2 high affinity receptor complex. Following three washes with FACS buffer, IL-2Rα\(^+\) CD8\(^+\) T lymphocytes were quantified by flow cytometric analysis.

13. **Chromium release assay**

The \(^{51}\)Cr release assay for the quantification of HSV-specific CTL was based on previously published methods (Bonneau, 1996; Bonneau et al., 1991a; Carter et al., 1981). Briefly, B6/WT-3 cells were infected with HSV-1 strain Patton. HSV- and mock-infected cells were then incubated at 37°C in the presence of 250 µCi of \(^{51}\)Cr in the form of sodium chromate (Na\(_2\)CrO\(_4\)); (PerkinElmer, Wellesley, MA) for 12-14 hours. The mock-infected cells were equally divided into two groups; one group served as a mock-
infected control and the other group was pulsed with 1 μM of gB498-505 peptide for 30 minutes at 37°C in 5% CO₂. Target cells (2.5 x 10⁴) were added in a volume of 100 μL to wells of a 96-well V-bottomed microtiter plate. An equal volume of effector cells was added at concentrations necessary to give the desired effector-to-target (E:T) cell ratios. In separate wells, equal volumes of ⁵¹Cr-labelled WT-3 cells and 5% (w/v) sodium dodecyl sulfate (SDS) were added to determine the maximum ⁵¹Cr release. The spontaneous ⁵¹Cr release was determined by combining equal volumes of ⁵¹Cr-labelled WT-3 cells and supplemented IMDM. The plates were centrifuged at 60 x g for five minutes and subsequently incubated for 4 hours at 37°C in 5% CO₂. The plates were then centrifuged at 60 x g for five minutes and 100 μL of the supernatant from each well were collected. A Cobra II gamma counter (PerkinElmer Life Sciences [Packard Instruments], Meriden, CT) was used to determine the counts per minute (cpm) in each sample. The mean of the three cpm values for each condition was obtained and used for all further analyses. The lytic activity for each E:T ratio was expressed as a percentage using the following equation:

\[
\text{Percent Specific Lysis} = \frac{\text{Experimental (cpm)} - \text{Spontaneous (cpm)}}{\text{Maximum (cpm)} - \text{Spontaneous (cpm)}} \times 100
\]

14. **Degranulation assay for T cell lytic function**

A modification of the method published by Betts and Koup was used for the detection of CD107a (LAMP-1) (Betts and Koup, 2004). Popliteal lymph node-derived lymphocytes were resuspended in supplemented IMDM and incubated with a 1:100 dilution of anti-CD107a FITC antibody (clone 1D4B; BD BioSciences Pharmingen, Cat. No. 553793)
and 1 µM of either gB498-505 or OVA257-264 peptide for 1 hour at 37°C. All cells were then treated with 10 mM of ammonium chloride to prevent acidification of endosomes and the subsequent loss of the FITC signal (Hoppe et al., 2004; Jin et al., 2005) and were incubated for an additional 3 hours at 37°C. Following this incubation, cells were washed twice with FACS buffer, and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were then incubated with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat, No. 551162), fixed in 2% paraformaldehyde, and analyzed by flow cytometric analysis.

15. **Enzyme-linked immunosorbent assay (ELISA)**

An enzyme-linked immunosorbent assay (ELISA) was used to quantitate the levels of IFN-γ and IL-2 in the lymphocyte culture supernatants from HSV-infected mice as described in detail elsewhere (Bonneau, 1996). Briefly, an IFN-γ (eBioscience, Cat. No. 14-7312-85) or IL-2 ‘capture’ antibody (BD Biosciences Pharmingen, Cat. No. 18161D) was added to a 96-well, flat-bottomed plate and incubated for overnight at 4°C. The residual IFN-γ or IL-2 ‘capture’ antibodies were then removed using PBS with 0.05% (vol/vol) Tween 20™ (polyoxyethylene-sorbitan monolaurate; Sigma-Aldrich) and the unbound sites on the plate were ‘blocked’ with 200 L of PBS/10%FBS for 2 hours at room temperature. Equal volumes of either culture supernatants or serial two-fold dilution of the appropriate cytokine standard (eBioscience) were added to the wells. After an overnight incubation at 4°C, wells were washed three times using PBS/Tween™
followed by the addition of 100 µL of biotin-conjugated anti-mouse IFN-γ (eBioscience, Cat. No. 13-7311-85) or anti-mouse IL-2 ‘detection’ antibody (BD Biosciences Pharmingen, Cat. No. 18172D). After a 45-minute incubation at room temperature, the wells were washed three times using PBS/Tween™ followed by a 30-minute incubation at room temperature with 100 µL of avidin peroxidase (2.5 µg/mL); (Sigma-Aldrich). The wells were washed three times with PBS/Tween™ followed by the addition of 100 µL of ABTS substrate (2,2’-Azino-bis(3-ethylbenthiazoline-6-sulfonic acid 0.3 mg/mL; Sigma-Aldrich) to each well. The optical density at 405 nm of each well was determined after 15 minutes using a Dynex Technologies MRX reader and Revelation v4.22 software (Dynex Technologies, Chantilly, Virginia).

16. Intracellular cytokine staining

To determine the percentage of CD8⁺ T cells that produced IFN-γ, 4 x 10⁶ lymph node-derived cells were resuspended in supplemented IMDM and incubated for 6 hours at 37°C in 5% CO₂ with either 1 µM gB₄⁹₈-₅₀₅ (SSIEFARL) peptide, OVA₂₅⁷-₂₆⁴ (SIINFEKL) peptide, or supplemented IMDM. Two hours after the start of this incubation period, cytokine secretion was prevented by blocking trans-golgi transport with brefeldin A at a final concentration of 5 µg/mL (Sigma-Aldrich). At the end of this 6-hour incubation period, cells were washed with FACS buffer and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were incubated, on ice and in the dark, with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat. No. 551162) and then washed
with FACS buffer/0.5% saponin (Sigma-Aldrich) to permeabilize the cell membrane. These cells were incubated for 20 minutes on ice with 50 µL of FITC-labeled anti IFN-γ antibodies (clone XMG1.2, eBioscience, Cat. No. 11-7311-82) diluted 1:100 in FACS buffer/0.5% saponin (Sigma-Aldrich). At the end of this incubation period, cells were washed three times with FACS buffer. Quantification of IFN-γ producing CD8+ T lymphocytes was determined using flow cytometric analysis.

17. **Flow cytometry analysis**

Flow cytometric analysis was conducted using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA). Using forward-angle light scatter and 90° light scatter profiles, electronic gates were set around the live cells and either 50,000 or 100,000 events were collected per sample. Dot plots and histograms were analyzed using FlowJo Software 6.4.7 (TreeStar, Inc.; Ashland, OR).

18. **Statistical analysis**

Statistical significance was determined by analysis of variance (ANOVA) using StatView 5.0.1 software (SAS Institute Inc, Cary, NC). The percentage cumulative survival was determined by Kaplan-Meier Cumulative Survival analysis using JMP 6.0.2 (Statistical Discovery, Cary, NC). Comparisons between groups were performed using unpaired t-test and p values < 0.05 were considered significant.
D. Results

1. Exogenous corticosterone elevates maternal and neonatal serum corticosterone levels

Choosing a model by which to chronically increase levels of maternal corticosterone was an important consideration in the design of these studies since the ability of the mothers to care for their neonates (e.g. nursing, warmth) needed to remain intact. For this reason, we chose to administer corticosterone in the drinking water to lactating mothers rather than attempting to use other chronic stress models such as restraint and electric footshock. Previous studies from our laboratory had already demonstrated that the administration of corticosterone in the drinking water increases maternal serum corticosterone levels and that withdrawal of this corticosterone results in a rapid return of serum corticosterone to baseline levels (Yorty et al., 2004).

Mothers were provided with corticosterone-supplemented water beginning on the day of delivery (day 0) and continuing for six additional consecutive days. On the morning of the sixth day, mothers were euthanized via cervical dislocation and blood was collected via cardiac puncture (Figure 2). Neonates were also euthanized and the blood and the contents of the neonate’s stomach were collected for subsequent analysis. As was expected, corticosterone in the drinking water significantly increased the mothers’ serum corticosterone levels as compared to their vehicle counterparts (Figure 3A; p < 0.01). These levels were approximately 8 times greater than baseline levels and were comparable to those increases that are observed using an acute maternal restraint/light stress model which we have previously used (Yorty et al., 2004). As was expected,
neonates nursing from mothers provided with corticosterone in the water exhibited increased levels of corticosterone in both the milk obtained from their stomachs (p < 0.001) and in their serum (p < 0.05) as compared to their vehicle counterparts (Figure 3B). Together, these findings demonstrate that the administration of corticosterone in the drinking water of lactating mice increases neonatal corticosterone levels and provides a means by which to determine the impact of maternally-derived corticosterone on the neonatal adaptive immune response.
Model Used to Determine The Effects of Exogenously Administered Corticosterone on Mothers and Neonates

200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days
Figure 3 (A and B)

**A.**

Maternal Serum Corticosterone (ng/mL)

- VEH: n=3
- CORT: n=5

- p < 0.01

**B.**

Neonatal Corticosterone (ng/mL)

- VEH Serum: n=8
- CORT Serum: n=20
- VEH Milk: n=8
- CORT Milk: n=16

- p < 0.05
- p < 0.001
Figure 3. Providing corticosterone in the drinking water increases the serum and milk corticosterone levels in mothers and their neonates. Lactating mothers were provided corticosterone (CORT)- (200 µg/mL) or vehicle (VEH)-supplemented water beginning on the day of delivery and continuing for six additional consecutive days. Levels of corticosterone in the maternal serum (A) and the neonatal serum and milk (B) were determined on the morning of day 6. Values represent mean ± SEM.
2. Neonatal exposure to maternally-derived corticosterone increases hind-limb paralysis and mortality post HSV-1 infection

Several studies have already described the impact of psychological stress, administered in the form of physical restraint, on HSV-induced hind-limb paralysis and mortality in adult mice (Anglen et al., 2003; Nair and Bonneau, 2006). However, the impact of maternally-derived corticosterone on neonatal hind-limb paralysis and mortality had yet to be determined. Therefore, we sought to determine the impact of neonatal exposure to maternally-derived corticosterone on the neonate’s morbidity and mortality post HSV-1 infection.

Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and monitored for up to thirty days post infection (Figure 4). Using this model, neonatal exposure to maternally-derived corticosterone was shown to increase the incidence of hind-limb paralysis (Figure 5A). The percent hind-limb paralysis for neonates that nursed from mothers with access to corticosterone-supplemented water beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group) was 73% and 47%, respectively. In addition, the survival of neonates in the 6-12 group that nursed from mothers with access to corticosterone-supplemented water was only 34%, a percentage that is significantly lower than for their vehicle counterparts (89%); (Figure 5B; p < 0.0001). These findings clearly demonstrate that neonatal exposure to
maternally-derived corticosterone increases the morbidity and mortality that is associated with neonatal HSV-1 infection.
Model Used to Determine the Effects of Maternally-Derived Corticosterone on the Neonatal Mortality Associated With HSV-1 Infection

200 μg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days

Infect pups in the rear footpads w/HSV-1

Monitor
Figure 5 (A and B)

A. Percent Neonatal Hind-Limb Paralysis

Maternal Exposure to Corticosterone (Days Post-Delivery)

VEH  n=40

CORT  n=43

VEH  n=29

CORT  n=37

B. Cumulative Percentage Survival

Days Post-Infection

VEH  n=51

p < 0.0001

CORT  n=66
Figure 5. Exposure of neonates to maternally-derived corticosterone increases the morbidity and mortality associated with HSV-1 infection. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group). At twelve days of age, neonates were infected in both rear footpads with HSV-1 Patton and were monitored for the development of hind-limb paralysis (A). Neonates were monitored for mortality for up to 30 days post-infection (B). Kaplan-Meier survival analysis was used to determine the cumulative percentage survival and the significance of any differences between the treatment groups.
3. **Neonatal exposure to maternally-derived corticosterone does not alter the splenic T cell profile**

To begin to examine the impact of maternally-derived corticosterone on the neonatal T cell component of the adaptive immune response, we first determined the impact of maternally-derived corticosterone on the neonatal splenic T lymphocyte profile. Because the lymph nodes are very small, they are unable to be located and removed from 12 day-old mice. Therefore, the spleen, being the largest single lymphoid organ in mammals and containing up to 25% of the body's mature lymphocytes (Brown, 1992), was chosen as the lymphoid organ of choice to determine the effect of maternally-derived corticosterone on the neonatal T cell profile.

Beginning on either the day of delivery (day 0) or six days post-delivery (day 6), mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented water for six consecutive days. Neonates were euthanized at twelve days of age and the splenic cellularity and the percentage of spleen-resident CD4\(^+\) and CD8\(^+\) cells was assessed (Figure 6). It had been documented both by our laboratory (Bonneau, 1996; Bonneau et al., 1991a) and others (Ehrich et al., 2004; Pruett et al., 2000a; Pruett et al., 2000b, 2003) that exposure of adult mice to stress, in the form of restraint, causes a substantial loss of lymphoid cells from the spleen. However, there had yet to be any reports describing the effects of maternally-derived corticosterone on the lymphoid composition of the neonatal spleen. As expected, the total number of splenic-derived lymphoid cells was markedly reduced in the neonates that nursed from mothers with access to corticosterone-supplemented water as compared to their vehicle counterparts.
(Figure 7A). This reduction occurred regardless of when the corticosterone was first made available to the mothers (either day 0 or day 6) and was not selective for cells of either the CD4+ or CD8+ T cell subsets as the percentages of these subsets in the spleen were similar in both sets of neonates (Figure 7B). The lack of a difference in the percentage of CD4+ or CD8+ cells, in light of our observed increase in HSV-associated morbidity and mortality, prompted us to further examine cell-mediated immune functions in these neonates since the spleen represents only a subset of the cells that are available to respond to an antigenic challenge.
Model Used to Determine the Effects of Maternally-Derived Corticosterone on the Neonatal Splenic Cellularity

200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days

Harvest Spleens
Figure 7 (A and B)

**A.**

```
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<tr>
<td>6-12</td>
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Number of Lymphoid Cells (x $10^6$)

Maternal Exposure to Corticosterone (Days Post-Delivery)

$\text{p} < 0.005$

**B.**

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Percentage of Total Lymphoid Cells

Maternal Exposure to Corticosterone (Days Post-Delivery)
Figure 7. Maternally-derived corticosterone reduces overall neonatal spleen cellularity without affecting the percentages of CD4$^+$ or CD8$^+$ spleen-resident cells. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group) and continuing for six additional consecutive days. At twelve days of age, neonates were euthanized and spleens were collected and processed as described in the Materials and Methods. The number of viable cells was determined using trypan blue dye exclusion (A) and the percentages of CD4$^+$ and CD8$^+$ cells were determined by flow cytometric analysis (B). Values represent mean ± SEM.
4. Neonatal exposure to maternally-derived corticosterone reduces the percentage of neonatal gB498-505-specific T cells generated in response to HSV-1 infection

The vast majority of the CTL generated in response to HSV-1 infection in C57BL/6 mice recognize the gB498-505 immunodominant peptide in the context of the H-2Kb MHC class I molecule of virus-infected cells (Bonneau et al., 1993b; Hanke et al., 1991; Nugent et al., 1994). Although several studies have already determined the impact of restraint stress on the generation of gB498-505-specific CTL in adult mice (Anglen et al., 2003; Nair and Bonneau, 2006), there has been a lack of similar studies delineating the impact of stress and stress-associated hormones on the HSV-specific CTL response in neonates. Given the findings described above, it was important to determine the impact of maternally-derived corticosterone on the frequency of neonatal lymph node-derived gB498-505-specific CTL.

Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for six additional consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were infected in the rear footpads with HSV-1 and euthanized five days post-infection. Popliteal lymph node-derived cells were collected and cultured as described in the Materials and Methods. At the end of this three-day incubation period, cells were stained with PE-conjugated gB498-505 tetramer and analyzed using flow cytometry (Figure 8). Neonates that nursed from mothers with access to corticosterone-supplemented water from days 0-6 (Figures 9 A and B) and 6-12 (Figures 9 C and D) had
a lower percentage of CD8+gB498-505+ cells as compared to their vehicle counterparts. These findings demonstrate that early neonatal exposure to maternally-derived corticosterone hinders the generation of HSV-1 specific CTL that are specific for HSV-encoded immunodominant epitope.
Model Used to Determine the Effects of Maternally-Derived Corticosterone on Neonatal HSV-Specific Cell-Mediated Immunity

200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days

Pups infected with HSV-1 in the rear footpads

Organs harvested, cultured for 3 days or tested ex vivo

Analyzed cultured cells
Figure 9

A. days 0-6 VEH
B. days 0-6 CORT
C. days 6-12 VEH
D. days 6-12 CORT

CD8 vs \( gB_{498-505} \)

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<th>( \text{CD8} )</th>
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Figure 9. Maternally-derived corticosterone reduces the percentage of CD8^{+} gB_{498-505}^{+} cells in neonates. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group; A, B) or six days (6-12 group; C, D), and continuing for six additional consecutive days. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and were euthanized five days post-infection at which time the popliteal lymph node-derived cells were cultured as described in the Materials and Methods. At the end of the 3-day culture period, the percentage of CD8^{+} gB_{498-505}^{+} cells was determined using flow cytometry. The results are presented as contour plots and are representative of two independent experiments.
5. **Neonatal exposure to maternally-derived corticosterone does not alter the percentage of early or late apoptotic lymph node-derived cells but does hinder the proliferation of splenic-derived T cells**

The reduced number of T lymphocytes described above could be attributed to either an increased level of apoptosis or a decreased proliferative ability of these cells. To distinguish between these two possibilities, the following studies were performed. Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for six additional consecutive days, mothers of neonatal mice were provided with either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, spleens were collected as described in the Materials and Methods (Figure 6). The translocation of phosphatidylserine (PS) in the plasma membrane from the inner to the outer leaflets marks the beginning of apoptosis. This translocation makes PS available to the 35-36 kDa, Ca$^{2+}$-dependent phospholipid-binding protein annexin V whose affinity for binding PS is high. Later in the apoptotic process, the plasma membrane loses its integrity, the DNA begins to fragment, and chromatin begins to condense. This loss of plasma membrane integrity makes cellular DNA accessible to the nucleic acid dye, 7AAD. As visualized by fluorescent flow cytometry, cells that are annexin V-negative are considered live cells, while cells that are only annexin V-positive are considered to be cells in the early stages of apoptosis. Cells that are both annexin V- and 7AAD-positive are either in the late stages of apoptosis or are dead. When the lymph node-derived cells from neonates that nursed from mothers with access to corticosterone-supplemented water were compared to their vehicle counterparts, no significant differences were
observed in the percentages of either early- (annexin-V+/7AAD-) or late-apoptotic (annexin-V+/7AAD+) cells (Figure 10A). These findings demonstrated that exposure of neonates to maternally-derived corticosterone does not alter the percentage of early or late apoptotic T lymphocyte in the spleens of neonates.

One of the hallmarks of an effective T cell-based immune response is the ability of activated T cells to expand clonally following T cell receptor-mediated activation (Cose et al., 1997). To further assess the impact of maternally-derived corticosterone on neonatal T cell function, we determined the effect of this corticosterone on the proliferation of splenic-derived T cells. Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for six additional consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were euthanized, the spleens were collected, and single cell suspensions were prepared as described in the Materials and Methods (Figure 6). Splenic-derived lymphocytes were cultured with either concanavalin A (Con A) or phytohemagglutinin-M (PHA-M) for 3 days. Con A is a T cell receptor-dependent polyclonal mitogen whereas PHA-M is a T cell receptor-independent mitogen. Splenic-derived T lymphocytes from neonates that nursed from mothers who had access to corticosterone-supplemented water beginning on either the day of delivery (0-6 group) or six days post-delivery (6-12 groups) exhibited a reduced proliferative ability in response to either Con A (Figures 10 B and D) or PHA-M (Figures 10 C and E) as compared to their vehicle counterparts. Only 38.4% of the splenic-derived T lymphocytes from neonates that nursed from mothers who had access to corticosterone-supplemented water beginning on either the day of delivery (0-6 group)
proliferated when stimulated with Con A compared to 76.3% for their vehicle counterparts. Additionally, when the splenic-derived T lymphocytes were stimulated with PHA-M, only 42.2% of cells from corticosterone-treated neonates proliferated as compared to 80.6% for their vehicle counterparts. In the 6-12 group, 49% of the splenic-derived T lymphocytes from corticosterone-treated neonates proliferated when stimulated with Con A as compared to 67.5% for their vehicle counterparts. Finally, when splenic-derived T lymphocytes were stimulated with PHA-M, only 46% of the cells derived from corticosterone-treated neonates proliferated as compared to 82% for their vehicle counterparts. Similar data were obtained when splenic-derived T lymphocytes were stimulated for 3 days in vitro using the potent protein kinase C activator phorbol 12-myristate 13-acetate (PMA) and ionomycin, a compound that increases intracellular Ca\(^{2+}\) concentration (data not shown). Together, these data demonstrate that maternally-derived corticosterone impairs the proliferative capacity of T lymphocytes without affecting apoptosis and thus may contribute to the ability of neonates to generate a pathogen-specific T cell-based immune response. Knowing that the overall number of splenic resident cells was significantly lower in neonates exposed to maternally-derived corticosterone as compared to their counterparts, we sought to determine the underlying mechanism responsible for this reduction.
Figure 10 A

Maternal Exposure to Corticosterone (Days Post-Delivery)

0-6

VEH CORT VEH CORT VEH CORT VEH CORT
Early Apoptotic Late Apoptotic Early Apoptotic Late Apoptotic

6-12

Percentage of Total Cells
Figure 10 (B-E)
Figure 10. Maternally-derived corticosterone does not induce apoptosis but does reduce the proliferative ability of neonatal splenic-derived T cells. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group) and continuing for six additional consecutive days. At twelve days of age, neonates were euthanized and splenic-derived cells were stained with annexin V and 7AAD as described in the Materials and Methods (A). Splenic-derived cells from neonates in the two groups were stained with CFSE and cultured with either Con A (B and D) or PHA-M (C and E) as described in the Materials and Methods. After 3 days of culture, the proliferation profile was determined using flow cytometry. The darker line represents the proliferation of cells from neonates that nursed from mothers with access to corticosterone-supplemented water, while the lighter line represents the proliferation for their vehicle counterparts. The results are representative of two independent experiments.
6. Neonatal exposure to maternally-derived corticosterone reduces the production of IL-2 and the expression of IL-2Rα by lymph node-derived cells

The activation of T cells leads to the generation of stimulatory and co-stimulatory signals that promote the transcription of IL-2 and IL-2Rα genes. The former is secreted and the latter, together with the β and γ subunits, forms the IL-2 receptor complex. The secreted IL-2 binds the IL-2R complex, which initiates a signaling cascade ultimately resulting in T cell proliferation. Given our observation that splenic-derived T cells from neonates exposed to corticosterone had a reduced proliferative capacity, we determined the levels of IL-2 secreted by lymph node-derived cells during the course of HSV-1 infection. Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for an additional six consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were infected in the rear footpads with HSV-1 and euthanized five days post-infection. Popliteal lymph node-derived cells were collected and cultured for three days as described in the Materials and Methods (Figure 6). At the end of this three-day incubation period, the amount of IL-2 secreted into the culture supernatant by these cells was quantitated by ELISA. Cultures derived from neonates that nursed from mothers with access to corticosterone-supplemented water from days 0-6 and 6-12 contained significantly-reduced levels of IL-2 as compared to their vehicle counterparts (Figure 11A). Additionally, when tested ex vivo, the percentage of CD8+ IL-2Rα+ cells from neonates that were nursed by mothers with access to corticosterone-supplemented
water beginning 6 days post-delivery (6-12 group) was significantly reduced as compared to their vehicle counterparts (Figure 11B). Together, these findings suggest a possible mechanism by which neonatal exposure to maternally-derived corticosterone could hinder the proliferation of antigen-stimulated cells.
Figure 11 (A and B)

A. 

IL-2 (pg/mL)

VEH
n=20

CORT
n=17

0-6

VEH
n=19

CORT
n=15

6-12

Maternal Exposure to Corticosterone (Days Post-Delivery)

B. 

Percentage of CD8+IL-2Rα+ Cells

Vehicle
n=16

Corticosterone
n=12

p < 0.05
Figure 11. Maternally-derived corticosterone decreases the expression of IL-2Rα and the production of IL-2 by neonatal lymphoid cells in response to HSV infection. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group) and continuing for six additional consecutive days. At twelve days of age, neonates were infected with HSV-1 in the rear footpads and five days post-infection were euthanized via cervical dislocation at which time the popliteal lymph node-derived cells were cultured as described in the Materials and Methods. At the end of the 3-day incubation period, the levels of IL-2 were determined in the culture supernatant (A). The percentage of IL-2Rα⁺ cells was determined ex vivo for neonates in the 6-12 group (B). Values represent mean ± SEM.
7. Neonatal exposure to maternally-derived corticosterone reduces HSV-specific CTL lytic activity in response to HSV-1 infection

Upon encountering the appropriate viral antigen-specific stimulatory and co-stimulatory signals, a naive CD8\(^+\) T lymphocyte becomes activated, expands in number, and acquires lytic activity necessary for the destruction of virus-infected cells. CTL play a critical role in mounting an effective cell-mediated antiviral immune response as these cells are able to destroy virus-infected cells, thus inhibiting the ability of the virus to replicate (Anglen et al., 2003; Bonneau, 1996; Bonneau et al., 1991a; Leo and Bonneau, 2000a, b). Given the increased HSV-associated morbidity and mortality in the neonates that nursed from mothers with access to corticosterone-supplemented water, we hypothesized that the ability of a neonate to generate a CTL response to HSV infection may contribute to the factors leading to these observations.

Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for six additional consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were infected in the rear footpads with HSV-1 and euthanized five days post-infection (Figure 8). Popliteal lymph node-derived cells were collected and cultured in vitro to expand the number of HSV-specific CTL for detection by \(^{51}\)Cr release assay (Jones et al., 2000) as described in the Materials and Methods. Lymph node-derived CTL from neonates that nursed from mothers with access to corticosterone-supplemented water from days 0-6 exhibited a reduced lytic activity against both HSV-infected and gB\(_{498-505}\) peptide-pulsed target cells as compared to their vehicle counterparts.
A similar, but non-significant, reduction in lytic activity was observed when the cells were derived from neonates whose mothers received corticosterone from days 6-12 (Figure 12B). These findings indicate that early exposure of neonates to corticosterone hinders the generation of lymph node-derived HSV-specific CTL.

A necessary precursor of cytolysis involves the release of cytotoxic granules from the lysosomes within the CTL (Betts et al., 2003). This release involves a transient expression of the lysosomal associated membrane protein-1 (LAMP-1) on the plasma membrane which is captured using fluorescently-labeled antibodies. Beginning six days post-delivery (day 6), and continuing for six additional consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were infected in the rear footpads with HSV-1 and euthanized five days post-infection. Popliteal lymph node-derived cells were collected and cultured for CD107a detection as described in the Materials and Methods. Lymph node-derived CTL from neonates that nursed from mothers with access to corticosterone-supplemented water exhibited a reduced percentage of CD8+CD107a+ cells when compared to their vehicle counterparts (Figure 12C). Being a more sensitive and truly quantitative assay, the CD107a assay data clearly demonstrates that exposure to maternally-derived corticosterone hinders the generation of functional lymph node-derived HSV-specific CTL that are able to lyse their target infected cells.
Figure 12 (A-C)

A. days 0-6

Percent Specific Lysis

B. days 6-12

Effector-to-Target Cell Ratio

C. days 6-12

Percentage CD81Ab-CD107a+

p < 0.005

Vehicle Corticosterone

n=28 n=20
Figure 12. Maternally-derived corticosterone decreases the HSV-specific lytic activity by cells derived from neonatal mice. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-partum (6-12 group), and continuing for six additional consecutive days. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and were euthanized five days post-infection at which time the popliteal lymph node-derived cells were cultured as described in the Materials and Methods. At the end of the 3-day culture period, the percent lytic activity was measured using the $^{51}$Cr release assay (A and B). Additionally, the percentage of CD8$^+$CD107a$^+$ cells from neonates in the 6-12 group was determined using flow cytometry (C). Values represent mean ± the SEM. An * indicates $p < 0.05$. The results are representative of those obtained from three independent experiments.
8. Neonatal exposure to maternally-derived corticosterone reduces the production IFN-γ by HSV-specific CD8⁺ T lymphocytes

To further delineate the effects of neonatal exposure to corticosterone on the HSV-specific cellular immune response, we determined the levels of IFN-γ secreted by HSV-specific CD8⁺ T lymphocytes. Interferon-γ can play a number of roles in the overall defense against HSV infection. Beginning either on the day of delivery (day 0) or six days post-delivery (day 6), and continuing for an additional six consecutive days, mothers of neonatal mice were provided either corticosterone- or vehicle-supplemented drinking water. At twelve days of age, all neonates were infected in the rear footpads with HSV-1 and euthanized five days post-infection. Popliteal lymph node-derived cells were collected and cultured for three days as described in the Materials and Methods (Figure 8). At the end of this 3-day incubation period, the amount of IFN-γ secreted into the culture supernatant by these cells was quantitated by ELISA. Cultures derived from neonates that nursed from mothers with access to corticosterone-supplemented water from days 0-6 and 6-12 contained significantly reduced levels of IFN-γ as compared to their vehicle counterparts (Figure 13A).

Given the above findings, it was important to determine whether the observed reduction in IFN-γ production could be attributed to either a reduced number of IFN-γ-producing cells or a decreased production of IFN-γ on a per cell basis. To address this issue, an intracellular cytokine assay for IFN-γ was used as described in the Materials and Methods. As compared to their vehicle counterparts, neonates that nursed from mothers who had access to corticosterone in the drinking water beginning on either the day of
delivery (day 0; Figures 13B, 8C) or six days post-delivery (day 6; Figures 13D, 8E) demonstrated a reduced percentage of CD8$^+$IFN-γ$^+$ cells. Together, these findings demonstrate that neonatal exposure to corticosterone reduces the overall percentage of HSV-specific CD8$^+$ cells that are able to produce IFN-γ.
Figure 13 A

IFN-γ (ng/mL)

Maternal Exposure to Corticosterone (Days Post-Delivery)

A. $p < 0.05$

- Vehicle n=16
- Corticosterone n=36
- Vehicle n=12
- Corticosterone n=8
Figure 13 B-E

- B. days 0-6 VEH
- C. days 0-6 CORT
- D. days 6-12 VEH
- E. days 6-12 CORT
Figure 13. Maternally-derived corticosterone reduces the percentage of CD8^+IFN-γ^+ cells in neonates. CORT- or VEH-supplemented water was made available to nursing mothers beginning either on the day of delivery (0-6 group) or six days post-delivery (6-12 group), and continuing for six additional consecutive days. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and were euthanized five days post-infection at which time the popliteal lymph node-derived cells were cultured as described in the Materials and Methods. At the end of the 3-day culture period, the amount of IFN-γ secreted into the culture supernatant was measured using IFN-γ ELISA (A) and the percentage of CD8^+IFN-γ^+ cells was determined using flow cytometry (B-E). The flow cytometry results are presented as contour plots and are representative of three independent experiments. Values represent means ± SEM.
E. Discussion

The studies described herein demonstrate that neonatal exposure to maternally-derived corticosterone increases the morbidity and mortality that is associated with HSV infection. Using a variety of state-of-the-art immunological methods, we also demonstrated that this exposure reduced the number and hindered the function of HSV-specific CTL that are generated in response to a local HSV infection. The ability of CTL to destroy virus-infected cells, and thus limit the production of progeny virus, greatly depends on the generation of an adequate number of functional CTL from the naive, virus-specific T cell repertoire. Such CTL can play a critical role in not only the defense against HSV but also against other pathogens whose immune-based resistance is mediated by CTL. Our findings are significant in that they emphasize the potential impact of corticosterone on a neonate’s ability to generate protective immunity not only following natural exposure to pathogens but also in response to the many vaccinations that neonates typically receive.

1. Modeling the corticosterone/cortisol component of post-partum maternal stress

A number of environmental factors contribute to the increased levels of psychological stress observed in nursing mothers during the post-partum period (Gröer et al., 2002). In the studies described herein, we have used a well-established experimental system in which we modeled these stress-associated increases in corticosterone alone
by providing nursing mice with corticosterone in the drinking water (Catalani et al., 2000; Ehrich et al., 2004; Yorty et al., 2004). Importantly, this system is non-invasive, highly reproducible, and does not alter maternal behavior with respect to the care of their pups (e.g. nesting, nursing, and/or abandonment) during the time of corticosterone administration. We recognize that this model is somewhat limited in that it determines the impact of only a single neuroendocrine component of the physiological response to stress (corticosterone) on immune function. However, this approach also provides a strength to these studies since it not only allows us to focus on a single, well-established critical mediator of immune function in adult mammals, but also that cortisol is known in humans to be transferred from the mother to the neonate via the transmammary route (Gröer et al., 2002; Kelly and Coutts, 2000; Koldovsky, 1980). Thus, this model is clinically relevant and can be used to better understand the mechanisms underlying the relationship among stress, neuroendocrine activation, and susceptibility to infectious pathogens.

As we and others have demonstrated (Angelogianni and Gianoulakis, 1989; Schmidt et al., 2003; Walker et al., 1986; Yorty and Bonneau, 2004a), neonatal mice, younger than 14 days of age, do not exhibit a stress response as measured by activation of the HPA axis activation and the concomitant increase in corticosterone. Thus, the increases in neonatal serum corticosterone that we observed reflect solely the increased levels of corticosterone that are transferred via the milk from mother to pup via the transmammary route (Figure 3B). It is important to note that unlike rodents, human neonates are able to elicit their own stress response as measured by increases in serum cortisol. Therefore, our studies serve as a model to determine the effects of both
transmammary- and endogenously-acquired cortisol on human neonatal immune function. The HPA hyperresponsiveness in neonatal C57BL/6 mice allowed us to isolate the impact of glucocorticoid on T cell-mediated immunity from other “stress hormones” produced in response to stress.

2. The impact of corticosterone on pathogen-associated morbidity and mortality

Neonates may be exposed to a number of infectious pathogens, many of which can result in significant morbidity and mortality (Anglen et al., 2003; Bonneau et al., 1997; Jones, 1996; Nair and Bonneau, 2006; Rudnick and Hoekzema, 2002; Whitley, 2004; Yorty and Bonneau, 2003, 2004a, b; Yorty et al., 2004). Although maternally-derived immunity acquired both prenatally (via the placenta) and postnatally (via the milk) is effective in controlling many of these pathogens, the lack of a timely and effective adaptive immune response on the part of the neonate can be detrimental. Exactly how stress-induced activation of the neuroendocrine system affects the development of such a response is not well characterized. Thus, an understanding of the relationship among the nervous, endocrine, and immune systems is critical and forms the basis for the studies described herein.

HSV is well recognized for its ability to cause significant pathology in the human host. Although HSV infections typically remain localized at distinct epithelial sites during primary infection and in the trigeminal (oral infections) and dorsal root (genital infections) ganglia during latent infection, systemic infections are possible, particularly
under conditions of immunosuppression (Bonneau and Jennings, 1989; Igietseme et al., 1989; Larsen et al., 1984; Larsen et al., 1983; Mitchell and Stevens, 1996; Sethi et al., 1983). Using adult murine models, studies by us and others have clearly supported a role for stress (Bonneau et al., 1997; Bonneau et al., 1990; Bonneau et al., 1993c; Bonneau et al., 1991b; Glaser and Kiecolt-Glaser, 2005a) and stress-associated corticosterone (Bonneau et al., 1993c; Catalani et al., 2000; Pruett et al., 2003; Yorty and Bonneau, 2004a; Yorty et al., 2004) in mediating increased pathology associated with HSV infection. We have extended these studies to a neonatal model in which we have shown (Figure 5A) a clear relationship between increases in maternal corticosterone and the pathology that is associated with HSV infection, regardless of when the corticosterone was provided to the nursing mothers (0-6 and 6-12 groups). The corticosterone-induced increase in hind-limb paralysis that is associated with HSV infection is typically not observed in either adult or neonatal C57BL/6 mice, but is usually followed by neurological sequelae and, in the most severe cases, death, as is illustrated in Figure 5B. A whole-body histological examination of moribund HSV-infected neonate mice indicated that the HSV infection remains localized to the central nervous system and does not spread to other organs and tissues even in the most severe cases of infection (data not shown). It is important to emphasize that the effects of corticosterone on HSV-associated morbidity are still observed even if the maternal source of corticosterone (corticosterone-supplemented drinking water) is removed 6 days prior to the start of HSV infection (Figure 5A, day 0-6 group). Interestingly, this morbidity was even more pronounced in the group in which the infection was initiated immediately following the last day of corticosterone exposure (day 6-12 group). This finding may reflect not only a decrease in
HSV-induced T cell activation but also a depletion of mature, yet naive T cells that are able to respond to an HSV infection.

3. Corticosterone effects on the neonatal adaptive immune response

The adaptive immune response plays an important role in the control of many infectious pathogens via either natural infection or vaccination. Most murine-based studies of adaptive immunity have focused on those responses in adult animals, particularly those responses that are elicited following a viral infection. For example, using an adult mouse model of HSV infection we and others have shown that HSV-specific CD8+ T cells play a critical role in controlling the extent of viral replication in footpads (Blaney et al., 1998; Bonneau and Jennings, 1989) and, in turn, reducing the degree of latent virus colonization of the dorsal root ganglia (Bonneau and Jennings, 1989). More recently, we have also shown the importance of CD8+ T cells in mediating protection from HSV encephalitis (Anglen et al., 2003; Nair and Bonneau, 2006).

In contrast to adults, the study of adaptive antiviral immune responses in neonates is sparse. However, we took advantage of recent studies by others (Franchini et al., 2001) in which neonatal mice were shown to generate a CTL response to HSV footpad infection. By adapting this model to test the hypothesis put forth within, we showed that 12-day old neonatal mice are indeed quite capable of mounting a HSV-specific adaptive immune response in response to HSV-1 footpad infection (Figures 9, 11, 12, 13). Although we certainly do not exclude the possibility that one or more components of the neonatal innate immune response may be operative in mediating protection in our model
of HSV infection, we have clearly shown in other studies (Anglen et al., 2003; Leo and Bonneau, 2000a, b; Leo et al., 1998) that HSV-specific CD8+ T cells play a critical role in mediating protection from HSV infection.

Prior to determining the impact of materially-derived corticosterone on the neonatal HSV-specific response, we simply wanted to determine whether or not maternally-derived corticosterone could affect the number and repertoire of lymphocytes in neonatal mice. Previous studies have demonstrated that psychological stress, administered in the form of restraint, reduces the number of spleen-resident T lymphocytes (Bonneau et al., 1998) in adults. Therefore, it was important to determine if neonatal mice would also exhibit a reduction in the number and proliferative capacity of splenic-resident T cells in our model. As expected, we showed that maternally-derived corticosterone reduces the overall number of splenic-resident T cells (Figure 7A) without altering the percentages of CD4+ and CD8+ cells (Figure 7B) at 12 days of age. The effect on splenic cell number was seen even 6 days after the source of corticosterone was removed (day 0-6 group), suggesting that the impact of stress on immune function has the potential to persist after the source of the stressor is removed. These findings provided the impetus for assessing the effect of maternally-derived corticosterone on HSV-specific immunity even after the corticosterone is removed.

Our studies of the HSV-specific CTL response focused on CD8+ T cells with specificity for HSV-encoded immunodominant, H-2Kb-restricted CTL epitope gB498-505 (Bonneau et al., 1993a). Knowing this epitope provided us with the opportunity to quantify both numerically and functionally the immune response using state-of-the-art methods such as tetramer-based flow cytometry (Figure 9), intracellular cytokine staining
Knowledge of this gB498-505 CTL recognition epitope coupled with each of these methods allowed us to thoroughly investigate the functional differences between the day 6-12 vehicle-treated and corticosterone-treated groups of mice. Clearly, the degranulation (Figure 12C), ELISA (Figure 13A), and intracellular cytokine (Figure 13B) data showed a functional difference between these two treatment groups. Thus, the lack of a observed difference in cytolytic activity as quantified by ⁵¹Cr release (Figure 12B) was somewhat perplexing. However, the lack of a difference may simply be a function of the required 3-day incubation period needed for the expansion of CTL to allow for the detection of cytolytic activity by this method (Jones et al., 2000). Combined, these methods clearly demonstrated that neonatal exposure to maternally-derived corticosterone reduces the number and function (Figures 9, 11, 12, 13) of gB₄⁹₈-₅₀₅-specific CTL generated in response to local HSV infection.

4. Immunological mechanisms underlying a reduced HSV-specific CTL response

Glucocorticoids have been long recognized for their ability to induce apoptosis in lymphocytes and other cells of the immune system (Amsterdam and Sasson, 2002; Amsterdam et al., 2002; Herold et al., 2006; Wang et al., 2006). Thus, our initial hypothesis was that apoptosis was simply responsible for our observed decrease in HSV-specific CTL. However, as illustrated in Figure 10A, there were no significant differences in the percentages of early and late apoptotic cells between neonates that
nursed from mothers with access to corticosterone-supplemented water and their vehicle counterparts. The lack of apoptosis in this model may have been due to the route by which the corticosterone was acquired (orally) and/or the dose of and timing with which the corticosterone was acquired by the neonate.

Upon stimulation, a CD8$^+$ T cell becomes activated and undergoes clonal expansion to yield effector HSV-specific CTL that are able to destroy virally-infected cells. Therefore, disrupting the ability of virus-specific CD8$^+$ T cells to proliferate limits the ability of the host to clear infected cells resulting in an increase in progeny virus which potentially leads to the host’s demise. In light of the above findings, we assessed the lymphoproliferative ability of spleen-resident cells and determined that the reduction in the number of lymphocytes observed is attributed to the cell’s hindered proliferative ability (Figures 10B-E). Interestingly, this lymphoproliferative capacity was still diminished even though the cells were no longer in the presence of the increased in vivo levels of corticosterone (0-6 and 6-12 groups; Figures 10B-E) and even when the source of corticosterone had been removed for 6 days prior to cell harvest (0-6 group; Figures 10B and C). These findings emphasize that exposure to glucocorticoids can have long-lasting effects on lymphocyte function, and possibly increase susceptibility to infectious pathogens long after the source of increased levels of glucocorticoids (exogenous and/or endogenous) are removed.
5. The role of corticosterone and other neuroendocrine mechanisms in modulating neonatal adaptive immunity and susceptibility to HSV infection

Glucocorticoids diffuse freely through the plasma membrane and into the cytoplasm where they bind to their natural ligand, migrate into the nucleus, and exert their activity via transactivation (production of NF-κB inhibitor) or transrepression (suppression of IL-2 production) (Almawi et al., 2002; Almawi and Melemedjian, 2002a, b; Auphan et al., 1995; Scheinman et al., 1995). Since signaling through the IL-2 receptor promotes cellular proliferation, glucocorticoid-mediated suppression of IL-2 synthesis could explain our observed reduction in the proliferative ability of splenic-derived cells (Figure 10B-E) and reduced percentage of CD8+ gB498-505+ cells (Figure 9). We demonstrated that lymph node-derived cells from neonates that nursed from mothers with access to corticosterone-supplemented water produced not only less IL-2 (Figure 11A) but that the expression of the IL-2Rα complex was also significantly diminished (Figure 11B) as compared to the cells derived from their vehicle counterparts. This reduction in IL-2Rα expression would magnify the reduction in the production of IL-2, ultimately reducing the ability of T cells to proliferate.

Glucocorticoids mediate their anti-inflammatory effects by regulating the gene expression of various cytokines (Almawi et al., 2002; Almawi and Melemedjian, 2002b). Activated CTL are able to clear infections via a variety of mechanisms, including the production of IFN-γ. IFN-γ-mediated recruitment of additional CTL to sites of infection facilitates efficient pathogen clearance (Collazo et al., 2001; Pan et al., 2005). The reduced levels of IFN-γ into the culture medium we observed (Figure 13A), are in
agreement with previous in vitro reports describing the ability of glucocorticoids to regulate IFN-γ at the mRNA level (Almawi et al., 1991; Arya et al., 1984; Gessani et al., 1988).

In addition to their well-known immunomodulatory effects, glucocorticoids, via receptor-mediated mechanisms, also lead to the production of catecholamines that are capable of modulating immune responses even further (Jiang et al., 2006; Oberbeck, 2006). Studies are in progress to determine how corticosterone may interact with the sympathetic nervous system in our experimental model and the role that products of the sympathetic nervous system may play in mediating the immunomodulatory effects as a consequence of neonatal exposure to maternally-derived corticosterone.

6. Implications of the current findings for neonatal health and beyond

These findings demonstrate that the exposure of neonates to maternally-derived corticosterone reduces the percentage, function, and overall lymphoproliferative ability of HSV-specific CTL. Such observations begin to shed light on the possible contribution of stress-elicited glucocorticoids to the morbidity and mortality in the neonates who are infected in the perinatal period not only with HSV but also with other pathogens to which they may be exposed and for which no maternally-derived immunity has been obtained. However, one should note that human neonates are able to elicit their own neuroendocrine response to environmental stressors — stressors that elicit a myriad of peptides and hormones that also have the potential to modulate immune function in the vulnerable neonate.
The studies described herein have focused exclusively on immune function and protection during the neonatal period. However, the impact of neonatal exposure to glucocorticoids on the development of T cell-based immunological memory during this neonatal exposure to either natural infection or vaccination is critical in the long-term health of an individual. Studies are ongoing to determine the impact of neonatal exposure to corticosterone during a primary immune response on their ability to generate a memory CTL response — a response that could contribute to protection should the individual be challenged again with infectious HSV as a consequence of either infection re-exposure to HSV or reactivation of the latent virus within the host. These studies begin to highlight the importance of maternally-derived stress hormones on the neonate’s cell-mediate immune responses.
Chapter III: Maternally-derived corticosterone alters the neonatal adaptive immune response to and pathogenicity of herpes simplex virus (HSV) infection via the type II glucocorticoid receptor

A. Abstract

Psychological stress has long been known to alter various components of the immune response to infectious pathogens in a number of adult species. More recently, we have used murine models to determine the impact of maternal stress and increased levels of maternal corticosterone on the ability of nursing neonates to mount an effective immune response to HSV infection. Although we observed both a suppressed HSV-specific immune response and an increased HSV-associated mortality in neonates who obtained milk from mothers exhibiting stress-like levels of serum corticosterone, we had not determined if these observations resulted directly from the actions of this maternally-derived corticosterone or whether other neuroendocrine-derived peptides and hormones, either maternally or neonatally derived, were involved. To address this issue in the studies described herein, we used a type II glucocorticoid receptor antagonist (RU486) and administered corticosterone to neonates directly via oral gavage. Using both of these approaches, we extended our original findings by now demonstrating that corticosterone itself directly suppresses the number, function, and protective capacity of HSV-specific CTL via its interaction with the type II glucocorticoid receptor. These findings suggest that maternal psychological stress and the associated increase in glucocorticoids may be an important factor not only in determining a neonate’s ability to resist infection with
naturally acquired pathogens but also in the development of protective immunity in response to the many vaccines which human neonates receive.
B. Introduction

There is abundant evidence that many components of immune function can be significantly modulated by psychological stress reviewed in (Bonneau and Hunzeker, 2007; Dhabhar and McEwen, 2007; Wetherell and Vedarha, 2007). The precise effects of such stress on immune function is dependent on the mode, intensity, and duration of the stressor, and is mediated by a wide range of neuroendocrine-derived peptides and hormones. The hallmark of a classical stress response is the activation of the HPA axis, culminating in the production of corticosterone (cortisol in humans).

Several murine models have been utilized by our laboratory and others to study neuro-endocrine-immune interactions (Adriaan Bouwknecht et al., 2007; Catalani et al., 2000; DeLano and Mallery, 1998; Stone et al., 1997; Yorty and Bonneau, 2003). Although the ability of corticosterone/cortisol to modulate immune function in adults has been known for quite some time (Leo et al., 1998; Nair and Bonneau, 2006; Nair et al., 2007b), its ability to alter immune function in neonates when obtained via transplacental and transmammary routes has only recently begun to be explored (Yorty and Bonneau, 2003, 2004a; Yorty et al., 2004; Zahwa et al., 2008). These studies have shown that the levels of prenatal and postnatal corticosterone in pups are directly proportional to the levels of stress and corticosterone in pregnant and nursing mothers, respectively (Yorty and Bonneau, 2004a; Yorty et al., 2004). Importantly, we demonstrated that neonates nursing from mothers with increased levels of corticosterone exhibit decreased levels of maternally provided HSV-specific antibody (Yorty et al., 2004) and are hindered in their ability to generate an adaptive CTL-based immune response to neonatal HSV infection.
As a result, such neonates exhibit an increase in HSV-associated morbidity and mortality. Although there is a clear relationship between the levels of maternal corticosterone and the magnitude of neonatal HSV-specific CTL responses, the direct impact of corticosterone on the generation and function of these HSV-specific CTL was not known.

It is logical for one to assume that maternally-derived corticosterone is directly responsible for suppressing the neonatal HSV-specific CTL response through its binding to type I and/or type II glucocorticoid receptors on the variety of cells that are involved in the activation, proliferation, and function of these CTL. Such an assumption is based on studies by us (Bonneau et al., 1993c) and others (Freeman et al., 2007) in which a role for corticosterone in the generation of a HSV-specific CTL response in adult mice was clearly demonstrated. However, there are other possibilities. For example, corticosterone is able to induce the synthesis and release of epinephrine from adrenal medullary cells (Betito et al., 1992; Wong et al., 1992). Therefore, maternal corticosterone may mediate effects on CTL generation and function in neonates through maternally and/or neonatally provided epinephrine. Such a hypothesis is supported by findings that catecholamines such as epinephrine are able to affect T cells through their interaction with β-2 adrenergic receptors (Hadden et al., 1970) also reviewed by Sanders and Straub (Sanders and Straub, 2002).

To test the hypothesis that maternally-derived corticosterone plays a direct role in modulating the neonatal immune response to and pathogenesis of HSV infection, we utilized an experimental approach whereby the type II glucocorticoid receptor antagonist RU486 was administered to mothers. Using this approach, we demonstrated that the
administration of RU486 fully restores the ability of neonates to generate HSV-specific CTL and to resist the morbidity and mortality that is associated with increased maternal levels of corticosterone. By administering corticosterone directly to neonates via gavage, we were able to reproduce the suppressed CTL response and increased pathogenicity that was observed in neonates nursing from mothers with increased levels of corticosterone.
C. Materials and Methods

1. Animals and experimental design

Four- to six-week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a controlled-temperature room (22-25°C) with a 12:12 hour light/dark cycle (lights on 0700-1900) and were allowed one week to acclimate to these conditions before any experimental manipulations were performed. Standard rodent diet (Harlan Teklad, Cat. No. 2018) and water were made available ad libitum. At the end of the acclimation period, individual female mice were housed with one male C57BL/6 mouse. The breeding pairs were left undisturbed for 14 days, at which time the males were removed and females were housed one mouse per cage. Litters were typically comprised of five to eight neonates; litters with less than five neonates were augmented to seven neonates per mother, and litters of greater than seven were culled to seven neonates per mother on the day of birth. For each experiment presented herein, at least six litters were designated for each experimental manipulation (Corticosterone, Vehicle …etc). Popliteal lymph nodes samples were pooled from three siblings per litter. Spleen samples were not pooled. Data shown represents experiments performed on the same cells, i.e. if pooled cells were used they were divided equally among the experiments. All mice were handled in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and the National Institutes of Health.
2. **Cell cultures and media**

Lymphocyte cultures utilized Iscove's-modified Dulbecco's medium (IMDM); (Gibco, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 0.225% (wt/vol) NaHCO₃, 25 mM Hepes buffer, 20 µM 2-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate.

3. **Virus**

HSV-1 strain Patton virus stocks were prepared in Vero cells by infection at a multiplicity of infection (MOI) of 0.01 and virus titers were determined by plaque assay on Vero cells. Virus stocks were stored at -70°C.

4. **Synthetic peptides**

Synthetic peptides corresponding to the HSV-1 CTL recognition epitope gB₄₉₈-₅₀₅ (SSIEFARL) (Bonneau et al., 1993a) and ovalbumin amino acid residues 257-264 (OVA₂₅⁷-₂₆₄; SIINFEKL) were synthesized at the Penn State Hershey Core Reasearch Facilities by FMoc chemistry using an automated peptide synthesizer (9050) MilliGen PepSynthesizer. The purity and amino acid composition of each peptide was determined by HPLC tracing (Waters, a division of MilliGen). Peptide stock solutions were prepared
by solubilizing the lyophilized peptides in dimethyl sulfoxide (DMSO) and adjusting the concentration to 1 mM with non-supplemented RPMI 1640 medium.

5. Exogenous corticosterone administration and experimental design

Corticosterone was dissolved in 30% (wt/vol) 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, Cat. No. H107-100G; St. Louis, MO) at 37°C with repeated vortexing and diluted to 200 µg/ml in tap water. Female mice were provided water containing either 200 µg/ml corticosterone (MP Biomedicals, Cat. No. 101416; Solon, OH) or as a control, vehicle (0.6% HBC); (Dhabhar and McEwen, 1999), ad libitum beginning either six days post-delivery (day 6), and continuing for six consecutive days. Water was changed every other day. To minimize the potential impact of animal handling on immunological measures, the bedding in all cages was changed only at fourteen days post-delivery (Balcombe et al., 2004). At twelve days of age, neonates were infected in the rear footpads with HSV-1. Five days post-infection (day 17) neonates were euthanized via cervical dislocation and popliteal lymph nodes were collected, processed, and stained for appropriate cell-surface markers.

6. Pharmacological blockade of receptor antagonists

The glucocorticoid receptor antagonist RU486 (Sigma-Aldrich, Cat. No. M8046-1G), was dissolved in sterile sesame oil (MP Biomedical, Cat. No. 156621) and injected subcutaneously (s.c.) into the mothers at a dosage of 25 mg/kg of body weight. The first
injection of RU486 occurred 24 hours prior to the administration of corticosterone- or vehicle-supplemented water. Injections were repeated daily until the when the corticosterone- or vehicle-supplemented water was removed. The 25 mg/kg dose of RU486 used in the studies described herein has also been shown to be effective in C57Bl/6 mice (Dobbs et al., 1993; Leo and Bonneau, 2000b; Lyte et al., 1991) and other animal models (Gorenberg et al., 2005).

7. Quantification of serum RU486

For the detection and quantification of serum RU486, liquid chromatography-mass spectrometry/mass-spectrometry (LC-MS/MS) was used. LC-MS/MS combines the power of high performance liquid chromatography (HPLC) and mass spectrometry (MS). Mass spectrometry combined with the separation power of liquid chromatography provides exceptionally low detection limits enabling high sensitivity quantitation over a wide range of flow rates. Samples were prepared and analyzed by the Pennsylvania State Hershey Core Research Facilities using appropriate controls.

8. gB498-505 tetramer staining

For the detection and quantification of gB498-505 epitope-specific CD8+ T lymphocytes, cells were first washed twice with FACS buffer (PBS supplemented with 1% [v/v] FBS, 0.02% [w/v] sodium azide), and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86;
San Diego, CA) for 20 minutes on ice. Cells were then incubated for 30 minutes on ice with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat. No. 551162) and a PE-labeled tetramer. This tetramer, which binds to the H-2K\(^b\)-restricted, gB\(^{498-505}\) T cell receptor complex (Blaney et al., 1998), was provided by NIH Tetramer Facility at Emory University. To control for non-specific tetramer binding, cells derived from non-HSV infected neonates were used. Following three washes with FACS buffer, gB\(^{498-505}\) epitope-specific CD8\(^+\) T lymphocytes were quantified by flow cytometric analysis.

9. Degranulation assay for T cell lytic function

A modification of the method published by Betts and Koup was used for the detection of CD107a (LAMP-1) (Betts and Koup, 2004). Splenic-derived lymphocytes were resuspended in supplemented IMDM and incubated with a 1:100 dilution of anti-CD107a FITC antibody (clone 1D4B; BD Biosciences Pharmingen, Cat. No. 553793; San Diego, CA) and 1 \(\mu\)M of either gB\(^{498-505}\) or OVA\(^{257-264}\) peptide for 1 hour at 37\(^\circ\)C. All cells were subsequently treated with 10 mM of ammonium chloride to prevent acidification of endosomes and the subsequent loss of the FITC signal and were then incubated for an additional 3 hours at 37\(^\circ\)C. Following this incubation, cells were washed twice with FACS buffer, and the CD16/CD32 Fc\(\gamma\) receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were then incubated with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat, No. 551162), fixed in 2% paraformaldehyde, and analyzed by flow cytometry.
10. **Intracellular cytokine staining**

To determine the percentage of CD8\(^{+}\) T cells that produced IFN-\(\gamma\), 4 x 10\(^6\) splenic-derived cells were suspended in supplemented IMDM and incubated for 6 hours at 37°C in 5% CO\(_2\) with either 1 \(\mu\)M gB\(_{498-505}\) (SSIEFARL) peptide, OVA\(_{257-264}\) (SIINFEKL) peptide, or supplemented IMDM. Two hours after the start of this incubation period, cytokine secretion was prevented by blocking trans-golgi transport with brefeldin A at a final concentration of 5 \(\mu\)g/mL (Sigma-Aldrich). At the end of this 6-hour incubation period, cells were washed with FACS buffer and the CD16/CD32 Fc\(\gamma\) receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were incubated, on ice and in the dark, with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat. No. 551162) and then washed with FACS buffer/0.5% saponin (Sigma-Aldrich) to permeabilize the cell membrane. These cells were incubated for 20 minutes on ice with 50 \(\mu\)L of FITC-labeled anti-IFN-\(\gamma\) antibodies (clone XMG1.2; eBioscience, Cat. No. 11-7311-82) diluted 1:100 in FACS buffer/0.5% saponin (Sigma-Aldrich). At the end of the incubation period, cells were washed three times with FACS buffer. Quantification of IFN-\(\gamma\)-producing CD8\(^{+}\) T lymphocytes was determined using flow cytometric analysis.

11. **Quantification of serum corticosterone**

The levels of corticosterone in the serum were determined using a radioimmunoassay-based (RIA) kit (MP Biomedical, Cat. No. 07-120103) and were
calculated using a standard curve generated from standards containing 0-1000 ng/mL of corticosterone.

12. Flow cytometry analysis

Flow cytometric analysis was conducted using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA). Using forward-angle light scatter and 90° light scatter profiles, electronic gates were set around the live cells and either 50,000 or 100,000 events were collected per sample. Dot plots and histograms were analyzed using FlowJo Software 6.4.7 (TreeStar, Inc.; Ashland, OR).

13. Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) using StatView 5.0.1 software (SAS Institute Inc, Cary, NC). The percentage cumulative survival was determined by Kaplan-Meier Cumulative Survival analysis using JMP 6.0.2 (Statistical Discovery, Cary, NC). Comparisons between groups were performed using unpaired t-test and p values < 0.05 were considered significant.
D. Results

1. The type II glucocorticoid receptor antagonist RU486 is transferred from mother to neonate via the milk.

We had previously demonstrated that maternally derived corticosterone is not only readily transferred to the neonate via the milk but also decreases survival and the HSV-specific T cell-mediated adaptive immune response in HSV-1-infected neonates (Zahwa et al., 2008). To begin to determine if these effects are attributable to maternally derived corticosterone, we needed to develop a method by which to repeatedly administer RU486 to the neonates beginning one day prior to their receipt of milk-derived corticosterone. However, given that such neonates are only 5 days of age and weigh less than 2 grams on the first day of RU486 administration, it was necessary to develop a method, other than systemic daily injections, to administer RU486. We hypothesized that RU486 administered to a nursing mother may, like corticosterone, be transferred to a neonate via the milk.

To test the above hypothesis, subcutaneous injections of RU486 were administered daily to nursing mothers beginning five days post-partum and continuing for seven consecutive days. On the morning of the seventh day, blood was collected from both mothers and their neonates and the serum was analyzed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS); (Figure 14). As was expected, mothers exhibited detectable levels of RU486 (Figure 15). Moreover, RU486 was detected in the serum of neonates that nursed from mothers receiving RU486. These
findings demonstrate that RU486 is indeed transferred from mother to neonate via the milk and thus provides a means by which to administer RU486 to neonates to delineate the effects of corticosterone on neonatal immune responsiveness.
Figure 14

Model Used to Determine if RU486 is Transferred from Mother to Neonate Via the Milk

![Diagram showing Pups Age in Days and Collect Blood Samples with RU486/Veh markings.]
Figure 15

Serum RU486 (ng/mL Per Gram of Body Weight)

<table>
<thead>
<tr>
<th></th>
<th>Mother</th>
<th>Neonate</th>
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<tbody>
<tr>
<td>VEHICLE</td>
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<td>$n=8$</td>
</tr>
<tr>
<td>RU486</td>
<td>$n=6$</td>
<td>$n=8$</td>
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</table>

$p<0.05$
Figure 15. RU486 is transferred from mother to neonate via the milk. Subcutaneous injections of RU486 were administered daily to nursing mothers beginning five days post-partum and continuing for seven consecutive days. Levels of RU486 in the maternal and the neonatal serum were determined on the morning of day 7. Values represent mean ± SEM
2. **Blockade of the type II glucocorticoid receptor partially restores splenic cellularity**

We had previously demonstrated that psychological stress, applied in the form of physical restraint, reduces splenic cellularity in adult mice (Bonneau, 1996; Bonneau et al., 1997; Bonneau et al., 1990; Bonneau et al., 1993c). More recently, we also demonstrated that maternally derived corticosterone reduces splenic cellularity in neonates (Zahwa et al., 2008). Knowing that primary immune responses can be modulated via both glucocorticoid-dependent and glucocorticoid-independent mechanisms (Bonneau et al., 1993c), and having determined that RU486 is transferred to the neonate, we sought to focus initially on the impact of corticosterone on neonatal splenic cellularity.

Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At twelve days of age, neonates were euthanized and the spleens were collected and processed as described in the Materials and Methods (Figure 16). The splenic cellularity and the percentage of spleen-resident CD4$^+$ and CD8$^+$ cells was assessed via fluorescent flow cytometry. As in our previously published findings (Zahwa et al., 2008), the total number of splenic-derived lymphoid cells was reduced in neonates who nursed from mothers with access to corticosterone-supplemented water (Figure 17). However, the administration of RU486 only partially restored neonatal splenic cellularity. These findings suggest that splenic cellularity is
mediated only partially by type II glucocorticoid receptor mechanisms and that the type I glucocorticoid receptor and/or products of the glucocorticoid-mediated activation of the sympathetic nervous system are involved in corticosterone-mediated reductions in splenic cellularity.
Model Used to Determine the Effects RU486 on Neonatal Splenic Cellularity

![Diagram showing the model used to determine the effects of RU486 on neonatal splenic cellularity. The diagram illustrates the administration of 200 µg/mL corticosterone or vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days, followed by the harvest of spleens.]
Figure 17

Neonatal Spleen lymphocytes (x10⁶)

<table>
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<th>Group</th>
<th>n</th>
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<tbody>
<tr>
<td>VEHICLE</td>
<td>5</td>
</tr>
<tr>
<td>VEHICLE + RU486</td>
<td>8</td>
</tr>
<tr>
<td>CORT</td>
<td>10</td>
</tr>
<tr>
<td>CORT + RU486</td>
<td>11</td>
</tr>
</tbody>
</table>

*p < 0.05*
Figure 17. The reduction in the overall neonatal spleen cellularity is mediated partially by type II glucocorticoid receptor mechanisms. Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At twelve days of age, neonates were euthanized and the spleens were collected and processed as described in the Materials and Methods. The number of viable cells was determined using trypan blue dye exclusion. Values represent mean ± SEM.
3. **Administration of RU486 to nursing mothers enhances the survival of HSV-1-infected neonates.**

We previously demonstrated that HSV-1-infected neonates who nursed from mothers with access to corticosterone-supplemented water exhibited a significant increase in HSV-associated mortality (Zahwa et al., 2008). We had hypothesized that this increased mortality was attributable to the maternally derived corticosterone. We based this hypothesis on the fact that both maternal and neonatal serum corticosterone levels were elevated in the group that was exposed to corticosterone (Zahwa et al., 2008).

To test the above hypothesis, subcutaneous injections of RU486 were administered to nursing mothers beginning five days post-partum and continuing for seven consecutive days. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and monitored for up to 28 days (Figure 18). Confirming our previously published findings (Zahwa et al., 2008), the survival of neonates who nursed from mothers with access to corticosterone-supplemented water was significantly lower than neonates who nursed from mothers who were provided vehicle alone (Figure 19). The administration of RU486 to the mothers, and its subsequent transfer to the neonate, enhanced the survival of neonates who nursed from mothers with access to corticosterone-supplemented water. Although neuroendocrine-immune interactions are obviously not limited to contributions by only glucocorticoids, these findings strongly support the hypothesis that maternally derived
corticosterone decreases neonatal survival directly by corticosterone and type II glucocorticoid-receptor-based interactions.
Model Used to Determine the Effects of MDC on the Neonatal Mortality Associated With HSV-1 Infection

- Infect pups in the rear footpads w/HSV-1
- Monitor
- 200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days
- Pups Age in Days

Figure 18
Figure 19

Cumulative Percentage Survival vs. Days Post Infection for different treatment groups:
- CORT + RU486 (n=47)
- VEHICLE (n=51)
- VEHICLE + RU486 (n=52)

$p < 0.0001$
Figure 19. Administration of RU486 to nursing mothers enhances the survival of HSV-1-infected neonates. Subcutaneous injections of RU486 were administered to nursing mothers beginning five days post-partum and continuing for seven consecutive days. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At twelve days of age, neonates were infected in the rear footpads with HSV-1 and monitored for up to 28 days. Kaplan-Meier survival analysis was used to determine the cumulative percentage survival and the significance of any differences between the treatment groups.
4. **Administration of RU486 to nursing mothers blocks corticosterone-mediated suppression of gB\textsubscript{498-505}-specific T cell production in HSV-1-infected neonates.**

The vast majority of CTL that are generated in response to HSV infection recognize the HSV-1 H-2\(K^{b}\)-restricted immunodominant epitope gB\textsubscript{498-505} (SSIEFARL) (Bonneau et al., 1993a; Cose et al., 1995; Hanke et al., 1991). To determine the ability of RU486 to block maternally-derived corticosterone-mediated suppression of gB\textsubscript{498-505}-specific T cell production in HSV-1-infected neonates, an H-2\(K^{b}\)/gB\textsubscript{498-505}-specific tetramer was used in conjunction with fluorescent flow cytometry.

Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Five days post infection, popliteal lymph nodes were collected and processed as described in the Materials and Methods (Figure 20). As was expected, the absolute number (Figure 21A) and percentage (Figure 21B) of gB\textsubscript{498-505}-specific CD8\(^{+}\) T cells was significantly reduced in the neonates who nursed from mothers with access to corticosterone-supplemented water. Additionally, the mean fluorescent intensity (MFI) of the gB\textsubscript{498-505}-specific CD8\(^{+}\) T cell receptor (TCR) was also significantly reduced in the neonates who nursed from mothers with access to corticosterone-supplemented water (Figure 21C), suggesting that the level of TCR expression was affected by corticosterone. However, the absolute number, percentage,
and MFI of gB_{498-505} CD8^{+} cells in neonates who were exposed to maternally derived corticosterone and RU486 was greater than in their counterparts who were exposed to corticosterone alone (Figures 21A, B, and C). These findings demonstrate that this maternal corticosterone-mediated reduction in neonatal CTL activity is mediated entirely via type II glucocorticoid receptor-dependent mechanisms.
Model Used to Determine the Effects RU486 on Neonatal HSV-Specific T Cell-Mediated Immunity

- Pups infected with HSV-1 in the rear footpads
- Organs harvested, cultured for 3 days or tested ex vivo
- Analyzed cultured cells
- 200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days

Figure 20
Figure 21 (A-C)

(A) CD8⁺ gB498,505⁺ (x10⁶)

VEHICLE | CORT | CORT + RU486

p < 0.05

(B) CD8⁺ gB498,505⁺

VEHICLE | CORT | CORT + RU486

p < 0.05
C.  $p < 0.05$  $p < 0.05$

![Graph showing MFI CD8^+ gB498-505 with bars for VEHICLE, CORT, and CORT + RU486](image)
Figure 21. The reduction in the overall number, percentage, and MFI of CD8$^+$ gB$_{498-505}$ in the neonates is mediated by type II glucocorticoid receptor mechanisms. Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Five days post infection; popliteal lymph nodes were collected and processed as described in the Materials and Methods. At the end of the 3-day culture period, the number (A) and percentage (B) of CD8$^+$ gB$_{498-505}$ cells was determined using flow cytometry. Additionally, MFI was calculated for CD8$^+$ gB$_{498-505}$ (C). Vehicle n=12, Corticosterone n=8, and Corticosterone+RU486 n=9. Values represent mean ± SEM.
5. Administration of RU486 to nursing mothers restores functional capacity of gB_{498-505}-specific CD8^{+} T cells in HSV-1-infected neonates

The ability of CTL to lyse virus-infected cells, and thus eliminate the production of viral progeny, makes them crucial in reducing the extent of pathogenicity. CTL attain this lytic function through their release of apoptosis-inducing granzymes that are stored in lysosomes. During the release of these granzymes, the lysosomal-associated membrane protein-1 (LAMP-1) is briefly expressed on the cell surface. The release of these granzymes is quantified using fluorescently conjugated antibodies specific for LAMP-1 (CD107a) together with fluorescent flow cytometry.

Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Five days post infection, popliteal lymph nodes were collected and processed as described in the Materials and Methods (Figure 20). Confirming our previously published results (Zahwa et al., 2008), the absolute number (Figure 22A) and percentage (Figure 22B) of CD8^{+}CD107a^{+} cells was significantly lower in the neonates who were exposed to the maternally derived corticosterone. However, the absolute number and percentage of CD8^{+}CD107a^{+} cells in neonates who were exposed to maternally derived corticosterone and RU486 was greater than in their counterparts who were exposed to corticosterone alone.
Interferon gamma (IFN-γ), is secreted by many cell types of the immune system. The ability of IFN-γ to alter the transcription of as many as 30 genes results in a wide range of responses that are necessary for orchestrating components of antiviral immunity (Schroder et al., 2004). The observed reduction in the absolute number (Figure 22C) and percentage (Figure 22D) of CD8⁺IFN-γ⁺ cells in the neonates who were exposed to the maternally derived corticosterone confirmed our previously published results (Zahwa et al., 2008). However, the absolute number and percentage of CD8⁺IFN-γ⁺ cells in neonates who were exposed to both maternally derived corticosterone and RU486 was greater than in their counterparts who were exposed to corticosterone alone (Figure 22C, and D). Together, these findings demonstrate that the reduction in the function of HSV-specific CD8⁺ cells is indeed mediated via type II glucocorticoid-dependent mechanisms.
Figure 22 (A-D)

A.

B.
C. 

\[ \text{CD8}^+\text{IFN}-\gamma^+ (\times 10^6) \]

- **VEHICLE**
- **CORT**
- **CORT + RU486**

\[ p < 0.05 \]

D. 

\[ \text{CD8}^+\text{IFN}-\gamma^+ \]

- **VEHICLE**
- **CORT**
- **CORT + RU486**

\[ p < 0.05 \]
Figure 22. The reduction in the overall number and percentage of CD8^+IFN-γ^+ and CD8^+CD107a^+ T cells in the neonates is mediated by type II glucocorticoid receptor mechanisms. Beginning five days post-partum and continuing for seven consecutive days, subcutaneous injections of RU486 were administered to nursing mothers. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Five days post infection; popliteal lymph nodes were collected and processed as described in the Materials and Methods. At the end of the 3-day culture period, the number (A) and percentage (B) of CD8^+IFN-γ^+ T cells in the neonates was determined using flow cytometry. Additionally, the number (C) and percentage (D) of CD8^+CD107a^+ T cells in the neonates was also determined using flow cytometry. Vehicle n=12, Corticosterone n=8, and Corticosterone+RU486 n=9. Values represent mean ± SEM.
6. Administering corticosterone to neonates via gavage elevates serum corticosterone.

The findings in these (Figures 19, 21, and 22) and previous studies (Zahwa et al., 2008) demonstrate that neonates who nurse on mothers exhibiting elevated levels of corticosterone have a decreased immune response to and increased pathogenesis of HSV infection. The ability of maternally administered RU486 to be transferred to the neonate (Figure 15) and to block corticosterone-mediated increases in HSV-associated mortality (Figure 19) and decreases in HSV-specific immune function (Figures 21 and 22) strongly suggest that corticosterone mediates these effects directly via the type II glucocorticoid receptor. To further confirm a direct role for corticosterone, and to rule out a role for other neuroendocrine hormones that may have been induced by corticosterone in either the mother or neonate, corticosterone was administered directly to neonatal mice via the oral route.

At six days of age, neonates received either 1.5, 30, or 300 µg of corticosterone via gavage as described in the Materials and Methods. At each of these doses, the physical appearance, mobility, and growth of these neonates was indistinguishable from neonates who were exposed to maternally derived corticosterone. At both 8 and 24 hours post-gavage, trunk blood was collected and the levels of serum corticosterone were determined by radioimmunoassay as described in the Materials and Methods. At 8 hours post-gavage, neonates who received 300 µg of corticosterone exhibited serum corticosterone levels comparable to those observed in mice experiencing psychological stress in the form of physical restraint (Figure 23). By 24 hours post-gavage, serum
corticosterone levels had returned to baseline levels. A similar rate of return to baseline has also been observed upon the termination of restraint stress. The lower doses of corticosterone (1.5 and 30 µg) did not elevate serum corticosterone in the neonates to levels above background (data not shown). Together, these findings demonstrated that corticosterone can be administered directly to neonates via gavage, and that the serum levels of corticosterone attained are comparable to mice undergoing psychological stress.
Figure 23.

VEHICLE
n=7

8 Hours
300 μg Cort/Mouse
n=4

24 Hours
n=5

Corticosterone (ng/mL Serum)
Figure 23. Administering corticosterone to the neonates via gavage elevates neonatal serum corticosterone concentrations at 8 hours post-gavage. At six days of age, neonates received 300 µg of corticosterone via gavage as described in the Materials and Methods. At both 8 and 24 hours post-gavage, trunk blood was collected and the levels of serum corticosterone were determined by radioimmunoassay as described in the Materials and Methods. Values represent mean ± SEM.
7. Administering corticosterone to neonates via gavage decreases gB\textsubscript{498-505}\textsuperscript{+}, CD107a\textsuperscript{+}, and IFN-\gamma\textsuperscript{+} T cells in HSV-1-infected neonates.

The use of gavage provides a means to determine the direct impact of elevated serum corticosterone on the neonatal T cell-based adaptive immune response to HSV infection. Beginning at six days of age and continuing for six consecutive days, neonates received 300 µg of corticosterone via gavage as described in the Materials and Methods. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Five days post-infection, popliteal lymph nodes were collected and processed as described in the Materials and Methods. Neonates who received corticosterone via gavage had significantly lower absolute numbers (Figure 24A) and percentages (Figure 24B) of CD\textsuperscript{8+}gB\textsubscript{498-505}\textsuperscript{+}, CD\textsuperscript{8+}CD107a\textsuperscript{+} and CD\textsuperscript{8+}IFN\gamma\textsuperscript{+} T cells. These results further confirmed already-published findings (Zahwa et al., 2008), and those described herein, indicating that orally-obtained corticosterone alone, such as that obtained by neonates via their mother’s milk is able to suppress the neonatal CD\textsuperscript{8+} T cell response to HSV infection.
Figure 24 (A and B)

A. Popliteal lymph node-derived CD8+ CTL (x10^6)

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B. Popliteal lymph node-derived CD8+ CTL

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</table>

p < 0.05
Figure 24. Corticosterone administered to the neonates via gavage reduces the number and percentage of CD8^+gB498-505^+, CD8^+IFN-γ^+, and CD8^+CD107a^+ T cells in the neonates. Beginning at six days of age and continuing for six consecutive days, neonates received 300 µg of corticosterone via gavage as described in the Materials and Methods. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Five days post-infection, popliteal lymph nodes were collected and processed as described in the Materials and Methods. At the end of the 3-day culture period, the number (A) and percentage (B) of CD8^+gB498-505^+, CD8^+IFN-γ^+, and CD8^+CD107a^+ T cells in the neonates was determined using flow cytometry. Vehicle n=10 and Corticosterone n=10. Values represent mean ± SEM.
E. Discussion

1. Corticosterone enhances the neurovirulence and mortality associated with HSV-1 infection.

HSV travels via neurons to the brain and, in some cases, establishes an infection that results in herpes simplex encephalitis (HSE). HSE accounts for approximately 10-20% of all viral encephalitis cases. In adults and children greater than 6 months of age, the prevalence of HSV encephalitis is 1 in 250,000-500,000 with a mortality rate as high as 60%, even with aggressive treatment (Aurelian, 2005). Despite such treatment, many survivors are still affected with permanent, severe, and often debilitating neurological damage (Aurelian, 2005).

In addition to the studies described above, we performed necropsies on neonatal mice five days post-HSV-1 infection. In keeping with our model of neonatal exposure to corticosterone, a subset of these mice were exposed to maternally-derived corticosterone for six consecutive days and then infected with HSV-1. All mice, regardless of corticosterone exposure, exhibited focal lesions in the brain. Interestingly, such lesions have been shown to be associated with activated CTL (Hudson and Streilein, 1994). However, it is the ability of HSV to migrate to the brainstem that is critical for determining survival in this model of HSV infection. Our studies in which corticosterone-exposed neonates exhibited increases in HSV-1-associated mortality (Figure 19 and Zahwa et al., 2008) led us to speculate that these mice exposed to maternally-derived corticosterone prior to infection would be more susceptible to HSE.
To address this issue, medulla and ventral pons regions of the brainstem from corticosterone- or vehicle-exposed neonates were stained using hematoxylin and eosin and were examined by conventional light microscopy. As is illustrated in Figure 25, neonates who were exposed to maternally-derived corticosterone exhibited lymphocytic perivascular cuffing (black arrowheads) unlike mice who were not exposed to maternally-derived corticosterone (Vehicle) and who exhibited intact blood vessels without any perivascular cuffing (green arrowheads). HSV-associated neuronal pathology in corticosterone-exposed neonates was evident by the presence of both neuronophagia (yellow arrowheads), and edema (red arrowheads). This type of pathology was not observed in vehicle-exposed neonates as is illustrated by the presence of numerous intact neurons (white arrowheads). These findings are in agreement with previous studies (Mori et al., 2005) in which brain edema was shown to be the cause of death in animals infected intranasally with HSV-1.

The reduction in the number of HSV specific CTL (Figure 21) allows the virus to replicate to a level much higher than it would under baseline serum corticosterone levels (Ashcraft et al., 2008). Previously published studies from our laboratory demonstrated that neonatal exposure to maternally derived corticosterone increases the morbidity and mortality associated with HSV-1 infection (Yorty et al., 2004; Zahwa et al., 2008). The studies described herein demonstrate that blocking the glucocorticoid receptor using the antagonist RU486 restored survival (Figure 19). Combined, these findings confirm that corticosterone was responsible for the increased morbidity and mortality.
Figure 25

Corticosterone  

Vehicle

4x

100x
Maternally-derived corticosterone increases HSV-associated pathology in the brainstem of neonates. Beginning five days post-partum and continuing for six consecutive days, corticosterone-supplemented water was made available to the mothers. At twelve days of age, neonates were infected with HSV-1 in the rear footpads. Five days post-infection, neonates were anesthetized and perfused with formalin after which time the brains were collected and stored in formalin for 72 hours and then transferred to 70% ethanol. The brains were embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin. Digital light microscopy revealed extensive lymphocytic perivascular cuffing (black arrowheads), edema (red arrowheads), and neuronophagia (yellow arrowheads) in the brainstems of neonates who nursed from mothers with access to corticosterone-supplemented water. Neonates who nursed from mothers with access to vehicle-supplemented water exhibited blood vessels without perivascular cuffing (green arrowhead) and numerous intact neurons (white arrowheads).
2. Maternal-neonatal interactions shape the neonate’s immune response for life.

The physiological interactions between a mother and her offspring starts at conception, continues through delivery, and extends for many weeks, if not years, into the life of the offspring. Breastfeeding provides the neonate with initial nutrients and immune defense mechanisms that contribute to survival. There is evidence to suggest that these interactions between a mother and her neonate affect both humoral and cell-mediated components of the neonate’s immune response (Cramer et al., 1974; Fagoaga and Nehlsen-Cannarella, 2002; Field and Caspary, 1971; Russell, 1975). In the studies described herein, we support and extend these findings by determining the impact of maternally-derived corticosterone on the cell-mediated immunity response to a clinically relevant pathogen.

In some cultures, breastfeeding occurs for up to 24 months following delivery. One cannot underestimate the value of this breastfeeding as its benefits extend well into adulthood, and possibly for a lifetime (Hanson, 1998). Breastfeeding occurs at a time when the neonatal immune system is developing in order to provide an adequate defense against pathogens. Interestingly, ultrasound examination has shown that the thymi of neonates who were breastfed are twice the size of their counterparts who are not breastfed (Hasselbalch et al., 1996). Even though human neonates are totally dependent on their mother’s breast milk for nutrition during the first few weeks of life, only 70% of the proteins contained in this milk provide nutrients to the neonates — the remaining 30% of the proteins are for the sole purpose of immune defense (Hanson et al., 2003). However,
we have previously demonstrated that maternal corticosterone is also readily transferred from a mother to her neonate via the milk (Yorty and Bonneau, 2004a; Yorty et al., 2004; Zahwa et al., 2008).

At baseline levels, glucocorticoids bind predominantly to the type I glucocorticoid receptor, or mineralocorticoid receptor. However, at higher levels, glucocorticoids bind to the type II glucocorticoid receptor, resulting in not only negative feedback control of the HPA axis and a wide array of metabolic affects to restore homeostasis, but also immunosuppression (Chrousos, 1998). In addition to being approved by the United States Food and Drug Administration (FDA) for the termination of early pregnancy, several phase II and phase III clinical trials have recently addressed the use of mifepristone (glucocorticoid receptor type II antagonist; also known as RU486) for the treatment of psychotic major depression and Alzheimer's disease (DeBattista et al., 2006; Flores et al., 2006; Nihalani and Schwartz, 2007; Simpson et al., 2005). Despite the potential of administering mifepristone to nursing mothers, either to terminate a subsequent pregnancy or to potentially treat depression, there are no published reports describing whether or not this compound is excreted in the milk. Thus, the FDA advises nursing mothers who are using mifepristone to abstain from breastfeeding from 3 to 10 days after they stopped using this drug. In the absence of safety data describing how mifepristone is excreted, and because blockade of the type II glucocorticoid receptor restores glucocorticoid-mediated immunosuppression in adult mice (Leo and Bonneau, 2000b; Nair and Bonneau, 2006; Nair et al., 2007b), we first sought to determine if RU486 is transferred from a mother to her neonate via the milk (Figure 24), and
subsequently determine the impact of such blockade on the neonatal adaptive cell-mediated immune response to HSV-1 infection (Figures 19, 21, and 22).

The use of RU486 is not without caveats (Gaillard et al., 1984). In addition to its traditional use as a type II glucocorticoid receptor antagonist, RU486 is also able to bind to the progesterone receptor, thus blocking this hormone-signaling cascade. Moreover, RU486 does not selectively bind to the glucocorticoid receptor in T lymphocytes but also binds to glucocorticoid receptors in all nucleated cells, potentially interfering with glucocorticoid feedback mechanisms (Spitz and Bardin, 1993). It is worth noting that 21-hydroxy-6,19-oxidoprogesterone, a more specific glucocorticoid type II receptor antagonist, exists with very limited commercial availability and little documented use in animal models (Veleiro et al., 1995; Vicent et al., 1997).

3. **Corticosterone partially regulates lymphoid organ cellularity.**

Greater than 25% of mature T cells reside in the spleen, the largest single lymphoid organ in the body (Brown, 1992). Therefore, it was logical for us to first examine the spleen to determine the broad effects of corticosterone on overall secondary lymphoid tissue cellularity. Several studies from our laboratory and others demonstrated that elevated levels of glucocorticoids result in reduced splenic cellularity in adult (Bonneau et al., 1993c; Bonneau et al., 1998; Leo et al., 1998; Pruett et al., 2000a) and neonatal mice (Zahwa et al., 2008). The partial restoration of cellularity in the spleens of HSV-infected neonates who were exposed to RU486 in addition to corticosterone clearly confirmed a role for glucocorticoids in determining splenic cellularity but also led us to
hypothesize a role for sympathetic nervous system-mediated mechanisms (Figure 17) (Bonneau et al., 1993c; Leo and Bonneau, 2000a, b). In support of this hypothesis, we have demonstrated that providing nursing mothers with epinephrine-supplemented water reduced neonatal splenic cellularity to levels similar to what is observed when corticosterone-supplemented water was made available to nursing mothers (data not shown). Given that epinephrine is rapidly oxidized, we hypothesize that perhaps an epinephrine metabolite(s) is/are responsible for the effects that we observed.

4. **Corticosterone hinders the neonatal T cell-mediated immune response to HSV infection.**

CD8$^+$ T cells recognize virus infected cells via their TCR. The binding between the TCR and MHC class I/virus-encoded peptide complex on the surface of an infected cell initiates a cascade of events resulting in the elimination of the infected cell. This elimination is critical in preventing the production of viral progeny. Thus, interfering with the ability of a CTL to bind to a virus-infected cell could have devastating consequences. The marked reduction in the expression level of the gB$_{498-505}$-specific TCR in neonates who nursed from mothers with access to corticosterone-supplemented water (Figure 21) could explain some of our findings. The reduction in the expression of this TCR weakens the affinity between the virus infected cell and the CTL which, in turn, reduces the ability of CTL to lyse virally-infected cells (Tian et al., 2007). This reduced level of gB$_{498-505}$-specific TCR expression does not reduce the ability of CTL to produce inflammatory cytokines on a per-cell basis (data not shown). However, the marked
reduction in the number and percentage of HSV-specific CTL (Figures 21, 22) resulting from elevated levels of serum glucocorticoid leads to uncontrollable spread of the virus as is evident by increased viral titer at the site of infection (Ashcraft et al., 2008).

One can not rule out the possibility that glucocorticoids are affecting cells other than CD8⁺ T lymphocytes, which could influence the potency of the immune response even further (Fernandez et al., 2008). Additionally, it has been demonstrated that HSV infection itself can also modulate the immune response (Bener et al., 2007; Gill et al., 2006). Alternatively, one could also postulate that exogenous corticosterone administration is affecting maternal metabolism and hormone secretion which could subsequently affect overall neonatal health.

5. Elevated neonatal glucocorticoids hinder the T cell-mediated immune response to HSV-1 infection.

To further confirm a role for corticosterone in the suppression of the neonatal immune response to HSV-1 infection we administered corticosterone to the neonates via gavage. The neonates tolerated this approach well and were physically indistinguishable from those neonates who nursed from mothers with access to corticosterone-supplemented water. This approach enabled us to rule out the possibility that corticosterone administered to the mothers resulted in the production of some other immunomodulators that were transferred to the neonate via the milk. For example, glucocorticoids can act directly on adrenal medullary cells ultimately resulting in increased serum epinephrine (Kelner and Pollard, 1985; Wurtman and Axelrod, 1966).
Thus, it was possible that corticosterone administration to the mothers could result in elevated levels of epinephrine in the maternal serum, and that this epinephrine could be transferred to the neonates via the milk.

Administering corticosterone directly to neonates via gavage enabled us to directly examine the role of corticosterone on the neonatal HSV-1 specific T cell-mediated immune response. A proof of concept experiment demonstrated that corticosterone administered orally to the neonate indeed resulted in elevated neonatal serum corticosterone levels (Figure 23). In complete concordance with the results obtained from experiments in which corticosterone was obtained by neonates from their mother (Figure 21 and 13), oral administration of corticosterone resulted in a hindered immune response to HSV-1 infection (Figure 24). Interestingly, synthetic glucocorticoids are often administered postnatally to premature neonates suffering from respiratory distress and thus requiring mechanical ventilation (Anday and Conway, 2003). This glucocorticoid treatment significantly decreases the risk of chronic lung disease (Anday and Conway, 2003); however, in light of our studies, this treatment also comes with a cost of elevated serum glucocorticoid levels that have the potential to dampen T cell-mediated immunity generated in response to infectious pathogens.

6. Implications of current findings on neonatal health

The impact of neonatal exposure to maternally derived glucocorticoids on neonatal health and well-being is a matter that warrants further investigation. The effects of such exposure extend well beyond the time of initial glucocorticoid exposure and may possibly
extend throughout life (Catalani et al., 2000). Overwhelming evidence from several studies indicate that infants exposed to high concentrations of maternal glucocorticoids resulting from high maternal stress or depression, exhibit higher basal glucocorticoid levels, increased morning cortisol secretion, poor attention, worse outcome in the occurrence of bronchopulmonary dysplasia, and intraventricular hemorrhage (Halligan et al., 2007; Locke et al., 1997; Tu et al., 2007). However, the immune system is certainly not spared from the effects of these maternally derived glucocorticoids. A significant reduction in the antibody titer in response to routinely administered vaccinations such as Haemophilus influenzae, DTP (diphtheria, tetanus, and pertussis), meningococcal conjugate, and varicella vaccines was observed in neonates that received synthetic glucocorticoids, such as dexamethasone (Clarke et al., 2006; Robinson et al., 1999; Robinson et al., 2004; Verstraeten et al., 2003). In the studies described herein, we demonstrated a role for glucocorticoids on the T cell adaptive immune response to an infectious pathogen. Our findings shed some light on the potential mechanisms underlying the effects of maternally derived glucocorticoids on neonatal health with respect to a neonate’s ability to respond immunologically to not only infectious pathogens but also to vaccinations. The compelling evidence that the effects of maternal stress extend beyond that of the mother and also includes the infant may warrant a need for stress management throughout the postnatal period.
Chapter IV: Neonatal exposure to maternally-derived corticosterone impairs memory cytotoxic T lymphocytes generated in response to herpes simplex virus (HSV) infection

A. Abstract

We have long been interested in determining the impact of psychological stress on the ability of newborns to mount an effective pathogen-specific immune response. For many viral pathogens, an effective immune response includes the generation of primary CTL which enhance survival and serve as precursors for CTLm that protect the host against subsequent exposure to the same pathogen. Previously, we have demonstrated that maternally derived corticosterone hinders the primary immune response against HSV-1 infection, at least in part, via type II glucocorticoid receptor mediated mechanisms. In the current studies, we sought to determine the impact of maternally derived corticosterone on neonatal HSV-specific CTLm.

Knowing that the quality of the CTLm repertoire reflects the quality of the primary CTL response, we hypothesized that neonates with a hindered primary immune response, resulting from the exposure to stress-like levels of glucocorticoids, would also exhibit a hindered CTLm repertoire as adults. Using a variety of flow cytometry-based approaches, we demonstrated that the CTLm repertoire in mice that were exposed to elevated level of glucocorticoids as neonates contained fewer memory HSV-specific T cell. We extended these findings by administering a glucocorticoid receptor antagonist and demonstrated that these effects were mediated by type II glucocorticoid receptor mechanisms.
Understanding the impact of psychological stress on a neonate’s ability to overcome infection and generate an effective memory immune response that is able to conquer future encounters with the same pathogen is crucial for the development of effective vaccine strategies. These results, combined with previous data from our laboratory, support the notion that maternal stress has the potential to suppress a neonate’s ability to mount an effective immune response against a variety of infectious pathogens.
B. Introduction

Several regulatory processes exist within the immune system to promote self-monitoring and to prevent uncontrollable immune responses (Syvalahti, 1987). There is also an abundance of evidence suggesting that immune modulation is also achieved through external regulation such as stress (Bonneau and Hunzeker, 2007; Dhabhar and McEwen, 2007; Wetherell and Vedhara, 2007). Depending on the duration and intensity of stressor, neuroendocrine signals up/downregulate the immune system (Glaser and Kiecolt-Glaser, 2005b). Thus, the central nervous and endocrine systems interact in a bidirectional fashion with each being able to up/down-regulate the other based on the psychological and physical conditions (Antoni, 2003; Shanks et al., 1998).

The psycho-neuro-endocrine interactions form an intricate network where one’s response to the psychological stimuli is achieved by activating various components of the neurological system that exhorts synergistic and antagonistic effects on the immune system. Psychological stress is broadly defined as a perceived stimuli that alters the homeostatic state (Chrousos and Gold, 1992; Johnson et al., 1992; Sternberg et al., 1992b). The hallmark of a classical stress response involves the activation of HPA axis, a process defined as early as 1941 by Dr. Hans Selye. Since then, only few studies addressed the impact of various maternal psychological stressors and the increased serum and milk cortisol levels on neonatal innate and adaptive immune responses against pathogens.
The nutritional dependence of mammalian neonates makes them vulnerable to their mother’s psychological state and her endocrine hormones that are transferred to the neonate via the milk. Until recently, there has been a lack of studies examining the effect of these elevated maternal immunomodulatory hormones on their nursing offspring.

Previous studies from our laboratory demonstrated that maternally derived corticosterone (MDC) hinders a neonate’s ability to mount an effective primary immune response against HSV infection by reducing CTL lytic activity, IFN-γ production, and percentage of HSV-specific T cells (Zahwa et al., 2008). The immediate outcome of these alterations is a drastic reduction in survival of neonates exposed to this MDC. Having demonstrated that MDC reduces neonatal survival, we hypothesized that administering the glucocorticoid receptor antagonist RU486 would restore neonatal survival. Indeed, neonatal exposure to RU486 along with corticosterone restored their survival post-HSV-infection.

Approximately 90-95% of the activated T cells are eliminated after pathogen clearance via apoptosis leaving behind more cells that are able to respond to the pathogen than the naive precursors specific for the same pathogen that were in the repertoire prior to the infection. The remaining 5-10% of activated T cells retain the ability to recognize cells infected with the same pathogen they were primed against. Cells that retain this ability are known as memory cytotoxic T cells (CTLm). Upon encountering the same antigen to which they were primed against, CTLm proliferate and eliminate their target virus-infected cells, thus reducing the time needed to mount
a T cell-mediated immune response (Harty and Badovinac, 2008; van Lier et al., 2003).

Although the origin of the CTLm subsets is still debatable, there is a consensus is that there exists at least two subsets, the central memory CTL (Tcm) that are CD8\(^+\)CD62L\(^+\), and effector memory CTL (Tem) that are CD8\(^+\)CD62L\(^-\). The unique functions for each of these two subsets ensures the generation of an effective immune response upon re-infection. Therefore, altering these CTLm subsets may result in an attenuated immune response by increasing the time needed to respond during a secondary encounter with a pathogen. Based on the knowledge that memory CTL are committed during the primary immune response, we hypothesized that MDC not only hinders the primary cell-mediated immune response, but also hinders the CTL memory cells.
C. Materials and Methods

1. Animals and experimental design

Four to six-week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a controlled-temperature room (22-25°C) with a 12:12 hour light/dark cycle (lights on 0700-1900) and were allowed one week to acclimate to these conditions before any experimental manipulations were performed. Standard rodent diet (Harlan Teklad, Cat. No. 2018) and water were made available ad libitum. At the end of the acclimation period, individual female mice were housed with one male C57BL/6 mouse. The breeding pairs were left undisturbed for 14 days, at which time the males were removed and females were housed one mouse per cage. Litters were typically comprised of five to eight neonates; litters with less than five neonates were augmented to seven neonates per mother, and litters of greater than seven were culled to seven neonates per mother on the day of birth. For each experiment presented herein, at least six litters were designated for each experimental manipulation. Popliteal lymph nodes were pooled from three siblings per litter. Spleen samples were not pooled. Data shown represents experiments performed on the same cells, (i.e. if pooled cells were used they were divided equally among the experiments). All mice were handled in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and the National Institutes of Health.
2. Cell cultures and media

Lymphocyte cultures utilized Iscove's-modified Dulbecco's medium (IMDM); (Gibco, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 0.225% (wt/vol) NaHCO₃, 25 mM Hepes buffer, 20 µM 2-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate.

3. Virus

HSV-1 strain Patton virus stocks were prepared in Vero cells by infection at a multiplicity of infection (MOI) of 0.01 and virus titers were determined by plaque assay on Vero cells. Virus stocks were stored at -70°C.

4. Synthetic peptides

Synthetic peptides corresponding to the HSV-1 CTL recognition epitope gB₄₉₈–₅₀₅ (SSIEFARL) (Bonneau et al., 1993a) and ovalbumin amino acid residues 257-264 (OVA₂₅₇-₂₆₄; SIINFEKL) were synthesized at the Penn State Hershey Core Research Facilities by FMoc chemistry using an automated peptide synthesizer (9050) MilliGen PepSynthesizer. The purity and amino acid composition of each peptide was determined by HPLC tracing (Waters, a division of MilliGen). Peptide stock solutions were prepared by solubilizing the lyophilized peptides in dimethyl sulfoxide (DMSO) and adjusting the concentration to 1 mM with non-supplemented RPMI 1640 medium.
5. **Exogenous corticosterone administration and experimental design**

Corticosterone was dissolved in 30% (wt/vol) 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma-Aldrich, Cat. No. H107-100G; St. Louis, MO) at 37°C with repeated vortexing and diluted to 200 µg/ml in tap water. Female mice were provided water containing either 200 µg/ml corticosterone (MP Biomedicals, Cat. No. 101416; Solon, OH) or as a control, vehicle (0.6% HBC); (Dhabhar and McEwen, 1999), *ad libitum* beginning either six days post-delivery (day 6), and continuing for six consecutive days. Water was changed every other day. To minimize the potential impact of animal handling on immunological measures, the bedding in all cages was changed only every fourteen days post-delivery (Balcombe et al., 2004). At twelve days of age, neonates were infected in the rear footpads with HSV-1. Thirty days post-infection neonates were euthanized via cervical dislocation and spleens and popliteal lymph nodes were collected, processed, and stained for appropriate cell-surface markers.

6. **Pharmacological blockade of receptor antagonists**

The glucocorticoid receptor antagonist RU486 (Sigma-Aldrich, Cat. No. M8046-1G), was dissolved in sterile sesame oil ((MP Biomedical, Cat. No. 156621) and injected subcutaneously (s.c.) into the mothers at a dosage of 25 mg/kg of body weight. The first injection of RU486 occurred 24 hours prior to the administration of corticosterone- or vehicle-supplemented water. Injections were repeated daily until the time when the
corticosterone- or vehicle-supplemented water was removed. The 25 mg/kg dose of RU486 used in the studies described herein has also been shown to be effective in C57BL/6 mice (Dobbs et al., 1993; Leo and Bonneau, 2000b; Lyte et al., 1991) and other animal models (Gorenberg et al., 2005).

7. Staining for memory CD8+ T lymphocytes

Central memory (Tcm) and effector memory (Tem) T lymphocyte subsets were identified using anti-CD62L APC (clone MEL-14; eBioscience; San Diego, CA). Following washes with FACS buffer (PBS supplemented with 1% [v/v] FBS, 0.02% [w/v] sodium azide), cells were resuspended in 2% (w/v) paraformaldehyde (prepared in PBS), Tcm and Tem subsets of gB498-505 epitope-specific CD8+ T lymphocytes were quantified by flow cytometric analysis.

8. gB498-505 tetramer staining

For the detection and quantification of gB498-505 epitope-specific CD8+ T lymphocytes, cells were first washed twice with FACS buffer (PBS supplemented with 1% [v/v] FBS, 0.02% [w/v] sodium azide), and the CD16/CD32 Fcγ receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86; San Diego, CA) for 20 minutes on ice. Cells were then incubated for 30 minutes on ice with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat. No. 551162) and a PE-labeled
tetramer. This tetramer, which binds to the H-2K\textsuperscript{b}-restricted, gB\textsubscript{498-505} T cell receptor complex (Blaney et al., 1998), was provided by NIH Tetramer Facility at Emory University. To control for non-specific tetramer binding, cells derived from non-HSV-infected neonates were used. Following three washes with FACS buffer, gB\textsubscript{498-505} epitope-specific CD8\textsuperscript{+} T lymphocytes were quantified by flow cytometric analysis.

9. Degranulation assay for T cell lytic function

A modification of the method published by Betts and Koup was used for the detection of CD107a (LAMP-1) (Betts and Koup, 2004). Splenic-derived lymphocytes were resuspended in supplemented IMDM and incubated with a 1:100 dilution of anti-CD107a FITC antibody (clone 1D4B; BD Biosciences Pharmingen, Cat. No. 553793; San Diego, CA) and 1 µM of either gB\textsubscript{498-505} or OVA\textsubscript{257-264} peptide for 1 hour at 37\degree C. All cells were subsequently treated with 10 mM of ammonium chloride to prevent acidification of endosomes and the subsequent loss of the FITC signal and were then incubated for an additional 3 hours at 37\degree C. Following this incubation, cells were washed twice with FACS buffer, and the CD16/CD32 Fc\gamma receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14z-0161-86) for 20 minutes on ice. Cells were then incubated with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat, No. 551162), fixed in 2% paraformaldehyde, and analyzed by flow cytometry.
10. **Intracellular cytokine staining**

To determine the percentage of CD8\(^+\) T cells that produced IFN-\(\gamma\), 4 x 10\(^6\) splenic-derived cells were suspended in supplemented IMDM and incubated for 6 hours at 37\(^\circ\)C in 5% CO\(_2\) with either 1 \(\mu\)M gB\(_{498-505}\) (SSIEFARL) peptide, OVA\(_{257-264}\) (SIINFEKL) peptide, or supplemented IMDM. Two hours after the start of this incubation period, cytokine secretion was prevented by blocking trans-golgi transport with brefeldin A at a final concentration of 5 \(\mu\)g/mL (Sigma-Aldrich). At the end of this 6-hour incubation period, cells were washed with FACS buffer and the CD16/CD32 Fc\(\gamma\) receptors blocked by incubating the cells with anti-CD16/CD32 antibodies (eBioscience, Cat. No. 14-0161-86) for 20 minutes on ice. Cells were incubated, on ice and in the dark, with anti-CD8 PerCP-Cy5.5 antibody (eBioscience, Cat. No. 551162) and then washed with FACS buffer/0.5% saponin (Sigma-Aldrich) to permeabilize the cell membrane. These cells were incubated for 20 minutes on ice with 50 \(\mu\)L of FITC-labeled anti-IFN-\(\gamma\) antibodies (clone XMG1.2; eBioscience, Cat. No. 11-7311-82) diluted 1:100 in FACS buffer/0.5% saponin (Sigma-Aldrich). At the end of the incubation period, cells were washed three times with FACS buffer. Quantification of IFN-\(\gamma\)-producing CD8\(^+\) T lymphocytes was determined using flow cytometric analysis.

11. **Flow cytometry analysis**

Flow cytometric analysis was conducted using a FACSCanto flow cytometer (Becton Dickinson, San Diego, CA). Using forward-angle light scatter and 90\(^\circ\) light scatter
profiles, electronic gates were set around the live cells and either 50,000 or 100,000 events were collected per sample. Dot plots and histograms were analyzed using FlowJo Software 6.4.7 (TreeStar, Inc.; Ashland, OR).

12. **Statistical analysis**

Statistical significance was determined by analysis of variance (ANOVA) using StatView 5.0.1 software (SAS Institute Inc, Cary, NC). The percentage cumulative survival was determined by Kaplan-Meier Cumulative Survival analysis using JMP 6.0.2 (Statistical Discovery, Cary, NC). Comparisons between groups were performed using unpaired \( t \)-test and \( p \) values < 0.05 were considered significant.
D. Results

1. Maternally derived corticosterone does not alter the percentage of neonatal memory CD8+ T lymphocytes generated in response to HSV infection

We have previously established that neonatal exposure to maternally derived corticosterone increased neonatal mortality and pathogenicity post-HSV-1 infection via mechanisms involving the adaptive immune system (Zahwa et al., 2008). Moreover, neonatal exposure to glucocorticoid receptor antagonist RU486 abrogates this increased mortality and HSV pathogenicity. Based on these findings, we sought to determine the impact of maternally derived corticosterone on the neonatal HSV-specific CTL memory repertoire. As infection is controlled, the activated T cell population begins to retract leaving behind a CTLm repertoire that is able to respond expeditiously to re-infection. This is a critical step as it prevents the constant presence of activated cells that are able to cause a wide array of autoimmune diseases (Harty and Badovinac, 2008; van Lier et al., 2003). Moreover, the persistence of CTLm repertoire significantly shortens the time needed for a robust response.

Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods (Figure 26).
We sought to determine the impact of maternally derived corticosterone on the neonatal overall CTLm population and its two subpopulations Tcm and Tem. When neonatal spleen homogenates were processed and stained using CD8 and CD62L fluorescently labeled antibodies and subsequently analyzed using flow cytometry, no differences were observed in the percentages of Tcm and Tem subpopulations between the 40-day-old mice who nursed from mothers with access to corticosterone-supplemented water and their vehicle counterparts (Figure 27). These findings demonstrate that even though there is a glucocorticoid-dependent generalized lymphocytopenia shortly after exposure to corticosterone, this does not affect the generation of CTLm and does not specifically target either the Tcm or the Tem subpopulations.
Model Used to Determine the Effects of Maternally-Derived Corticosterone on Neonatal HSV-Specific CTLm

- Pups infected with HSV-1 in the rear footpads
- 200 μg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days
- Organs harvested and cells analyzed ex vivo
Figure 27

Percentage of CD8+ Splenic-Resident CTLm Cells

- CD62L+ T_{CM}
- CD62L+ T_{EM}

Veh
Cort
Figure 27

Exposure to maternally derived corticosterone as a neonate does not reduce the percentage of either Tem or Tcm. Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods. The percentage of CTL memory cells was not different as compared to the CTLm repertoire in the spleens of mice who were exposed to either corticosterone or vehicle as neonates. Values represent mean ± SEM.
2. **Maternally derived corticosterone reduces the percentage of HSV-specific Tem**

Tem are poised to respond quickly to pathogenic re-encounter. These cells are able to expand in numbers to produce armed CTL that are able to clear infected cells (van Lier et al., 2003). Even though the overall percentage of Tem is similar in the spleens of neonates who nursed from mothers with access to corticosterone and their vehicle counterparts, we determined the impact of maternally derived corticosterone on the HSV-specific Tem.

Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods (Figure 26). We sought to determine the impact of maternally derived corticosterone on the neonatal Tem subpopulation that is able to recognize the HSV-1 immunodominant peptide in the context of MHC-I molecule on the surface of HSV-infected cells. The percentage of Tem that express the TCR specific for HSV-1 infected cells was significantly lower in the mice that nursed from mothers with access to corticosterone supplemented water as compared to their vehicle counterparts (Fig 28A). Moreover, the MFI of the gB\textsubscript{498-505}-specific CD8\textsuperscript{+} TCR on these Tem was also significantly reduced in the neonates who nursed from mothers with access to corticosterone-
supplemented water (Figure 28B). Together, these findings demonstrate that the reduction in the percentage of HSV-specific Tem and the reduction in the expression level of the HSV-1 specific TCR on the surface of Tem is mediated via glucocorticoid dependent mechanisms.
Figure 28 (A and B)

A.

B. 
Figure 28

Exposure to maternally-derived corticosterone as a neonate reduces the percentage of HSV-specific CTL and the HSV-specific T cell receptor expression level. Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. At thirty days post-infection, neonates were euthanized via cervical dislocation and these spleens were harvested and processed as described in the Material and Methods. The percentage CD8^+ gB_{498-505}^+CD62L^− CTLm in the spleens of animals who were exposed to corticosterone as neonates was significantly lower as compared to their vehicle counterparts (A). Additionally, the mean gB_{498-505} T cell receptor fluorescence intensity (MFI) was significantly lower in the cells derived from the spleens of animals who were exposed to corticosterone as neonates as compared to their vehicle counterparts (B). Values represent mean ± SEM.
3. Neonatal exposure to maternally derived corticosterone reduces HSV-specific Tem CTL lytic activity in response to HSV-1 infection

Effector memory CTL are poised to quickly respond to pathogens to which they have been activated against. These pathogen-specific CTL are able to expand clonally upon encountering infected cells, yielding a population that is able lyse these virus-infected cells and thus depriving the virus an opportunity to propagate. These memory CTL are maintained for the life as demonstrated in laboratory animal models (Homann et al., 2001). Therefore, it is necessary to determine the impact of maternally derived corticosterone on the pathogen-specific CTLm repertoire.

Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. At thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods (Figure 26). The ability of pathogen-specific cells to release the contents of their cytolytic granules is critical for inducing apoptosis in their target virus-infected cells. This release is accompanied with a brief expression of the lysosomal associated membrane protein-1 (LAMP-1). This brief expression is quantified using anti LAMP-1 fluorescently labeled antibodies. Using flow cytometry analysis, we observed that the percentage of splenic-derived CD8\(^+\)CD107a\(^+\) Tem was lower in the mice who nursed from mothers with access to corticosterone-supplemented water as compared to their vehicle counterparts (Figure 29). These findings clearly
demonstrate that reduction in the percentage of pathogen-specific Tem that are able to lyse their target virus-infected targets is mediated via glucocorticoid-dependent mechanisms.
Figure 29

Percentage of CD8<sup>+</sup>-Ab-CD62L<sup>-</sup>CD107α<sup>+</sup>

- Vehicle: 5%
- Corticosterone: 2%

$p < 0.05$
Figure 29

Neonatal exposure to maternally-derived corticosterone reduces the percentage of effector CTLm that are able to lyse virus-infected cells. Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. At thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods. The percentage CD8$^+$CD017a$^+$ CTLm in the spleens of animals exposed to corticosterone as neonates was significantly lower as compared to their vehicle counterparts. Values represent mean ± SEM.
4. Neonatal exposure to maternally derived corticosterone reduces the production IFN-γ by HSV-specific Tem CTL

IFN-γ, is secreted by many cell types of the immune system. The ability of IFN-γ to alter the transcription of as many as 30 genes results in a wide range of responses that are necessary for orchestrating components of antiviral immunity (Schroder et al., 2004). When effector CTL encounter infected cells, they rapidly produce IFN-γ. Thus, it is important to determine the impact of maternally derived corticosterone on the CTLm repertoire and the ability of cells in this repertoire to manifest their antimicrobial functions.

Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. Thirty days post-infection neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods (Figure 26). The ability of effector cells, stimulated ex vivo with the HSV-immunodominant peptide (gB<sub>498-505</sub>), to produce IFN-γ upon was assessed using fluorescently labeled IFN-γ antibodies in a standard intracellular cytokine staining assay. The percentage splenic-derived Tem that produced IFN-γ was significantly lower in neonates who nursed from mothers with access to corticosterone-supplemented water as compared to their vehicle counterparts (Figure 30). These findings demonstrate that indeed
glucocorticoid-mediated mechanisms are controlling the magnitude of pathogen-specific CTLm that are able to produce IFN-γ.
Figure 30

The graph shows the percentage of CD8^+CD62L^−IFNγ^+ cells in Vehicle and Corticosterone treatments. The black bar indicates the Vehicle group with a percentage of 5%, while the white bar represents the Corticosterone group with a percentage of 4%. The p-value is less than 0.05, indicating a statistically significant difference between the two groups.
Neonatal exposure to maternally-derived corticosterone reduces the percentage of effector CTLm that produce IFN-γ. Beginning five days post-partum, and continuing for six consecutive days, corticosterone- or vehicle-supplemented water was made available to nursing mothers. At 12 days of age, neonates were infected with HSV-1 in the rear footpads. At thirty days post-infection, neonates were euthanized via cervical dislocation, spleens were harvested and processed as described in the Material and Methods. The percentage CD8^+IFNγ^+ CTLm in the spleens of animals exposed to corticosterone as neonates was significantly lower as compared to their vehicle counterparts. Values represent mean ± SEM.
5. **Administration of RU486 to nursing mothers restores functional capacity of gB_{498-505}-specific Tem CD8^+ T cells in HSV-1-infected neonates**

We had previously demonstrated that psychological stress, applied in the form of physical restraint, reduces splenic cellularity in adult mice (Bonneau, 1996; Bonneau et al., 1997; Bonneau et al., 1990; Bonneau et al., 1993c). More recently, we also demonstrated that maternally derived corticosterone reduces splenic cellularity in neonates (Zahwa et al., 2008). The primary immune responses can be modulated via both glucocorticoid-dependent and glucocorticoid-independent mechanisms (Bonneau et al., 1993c). Moreover, having determined that RU486 is transferred to the neonate (previous data chapter), and that this modulation of the neonate’s primary immune response was mediated via the interaction between glucocorticoids and their receptor, we sought to determine if the restoration of the primary immune response is accompanied by a restored magnitude of the CTLm response.

Beginning four days post-partum, and continuing for seven consecutive days, mothers received daily subcutaneous injections of RU486 as described in the Materials and Methods. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Thirty days post-infection, neonates were euthanized via cervical dislocation; spleens were harvested and processed as described in the Material and
Methods (Figure 31). The percentage of CD8^+CD62L^{gB_{498-505}} (Figure 32A), CD8^+CD62L^{IFN-\gamma^+} (Figure 32B), and CD8^+CD62L^{CD107a^+} (Figure 32C) in the neonates who were exposed to both RU486 and maternally derived corticosterone was greater than their counterparts who were exposed to maternally derived corticosterone alone. Together, these findings further confirmed that the reduction in the percentage of CD8^+CD62L^+ HSV-specific cells previously reported in this data section is indeed mediated via type II glucocorticoid-dependent mechanisms.
Model Used to Determine the Effects of RU486 and Maternally-Derived Corticosterone on Neonatal HSV-Specific CTLm

- 200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days
- Pups infected with HSV-1 in the rear footpads
- Organs harvested and cells analyzed ex vivo

200 µg/mL corticosterone or Vehicle (2-Hydroxypropyl-β-cyclodextrin) for 6 days

Pups Age in Days
0 2 4 6 8 10 12 14 36 38 40 44

RU486/Veh
Cort/Veh

Figure 31
Figure 32 (A-C)

A.

B.
C.

Percentage of CD8^+CD62L^−CD107^a^+ Cells

- VEH:
- CORT:
- CORT+RU486:

*p < 0.05*
Neonatal exposure to both maternally-derived glucocorticoid receptor antagonist and corticosterone restores the magnitude of CTLm HSV-specific response in the mice who were exposed to maternally-derived corticosterone alone. Beginning four days post-partum, and continuing for seven consecutive days, mothers received daily subcutaneous injections of RU486 as described in the Materials and Methods. One day following the first injection of RU486, corticosterone-supplemented water was made available to the mothers for six consecutive days. At 12 days of age, neonates were infected in the rear footpads with HSV-1. Thirty days post-infection, neonates were euthanized via cervical dislocation; spleens were harvested and processed as described in the Materials and Methods. The percentage of CD8⁺CD62L⁻gB₄⁹₈⁻₅₀₅⁻ (A), CD8⁺CD62L⁻IFN-γ⁺ (B), and CD8⁺CD62L⁻CD107a⁺ (C) in the spleens of these mice was determined using flow cytometry. Values represent mean ± SEM.
E. Discussion

1. Glucocorticoid-mediated mechanisms impair the HSV-specific CTL memory response.

Previously published studies demonstrated that neonatal exposure to maternally derived corticosterone increases the morbidity and mortality that is associated with HSV infection (Yorty and Bonneau, 2004a; Yorty et al., 2004; Zahwa et al., 2008). Studies described herein demonstrate that corticosterone is not only able to hinder the neonatal T cell-mediated adaptive immune response during primary infection (Zahwa et al., 2008), but also hinder the memory CTL response the host depends on for protective immunity against future encounter with the same pathogen. Data describing the use of glucocorticoid receptor antagonist RU486 in the previous data chapter demonstrated that the neonatal T cell-mediated adaptive immune response during primary infection is mediated via the interaction between glucocorticoids and their type II glucocorticoid receptor. Data presented in this data chapter further demonstrated a role for glucocorticoids in modulating T cell-mediated adaptive immune response. The ability to restore the CTLm response using the glucocorticoid receptor antagonist RU486 further confirmed a role for glucocorticoid in modulating the CTLm response (Figure 32). Our findings emphasize the potential impact of corticosterone on a neonate’s ability to generate long-term protective immunity not only following natural exposure to pathogens but also in response to the many vaccinations that neonates typically receive.
2. **Glucocorticoids imprint the neonate’s immune response long after their removal.**

The adult mice who were exposed to stress-like levels of glucocorticoid while they were nursing, appeared physically indistinguishable from their vehicle counterparts. Overall, the adult mouse’s total body weight, fur coat appearance, general activities, food and water consumption, and overall appearance was independent of exposure to stress-like levels of glucocorticoids as neonates. Interestingly though, the CTL memory cell repertoire told a different story. This repertoire, which has not been exposed to elevated levels of glucocorticoid for over 28 days, still exhibited reduced efficiency evident by the reduced percentage of \( \text{CD}8^+\text{CD62L}^{-}\text{gB}_{498-505}^+ \) (Figure 28), \( \text{CD}8^+\text{CD62L}^{-}\text{IFN-}\gamma^+ \) (Figure 30), \( \text{CD}8^+\text{CD62L}^{-}\text{CD107a}^+ \) (Figure 29). This reduction could have devastating consequences as the presence of an effective memory pool protects the host from re-infection. The ultimate proof of concept experiment to test the protective ability of the CTLm repertoire would have been re-infecting the mice that were exposed to stress-like levels of glucocorticoid as neonates and monitor their survival. It is generally recognized that both humoral (antibody) and adaptive (T cell-mediated) immune responses are mounted against HSV-1 infection. Therefore, such an experiment would not tease apart the effects of the humoral antibody mediated protection from the focus of my studies, T cell-mediated adaptive immunity.
3. **Implication of glucocorticoid mediated hindered CTL memory immune response on the neonate’s health.**

The need for a neonate to mount an immune response begins during delivery where they begin encountering pathogens. This need continues for the individual’s entire life, as the continuous exposure to pathogens demands an immune response to ensure the host’s survival. It is also worth noting within days of delivery, neonates in the United States are immunized against Hepatitis B, an immunization that requires a potent immune response. These responses early in life are generated when the neonate is almost entirely dependent on the mother’s milk for nutrition and immune defenses. As mentioned earlier in the literature review, the mother’s milk contains critical antibodies needed for the neonate’s survival in the face of countless pathogen they are encountering. It is also worth noting that this mother’s milk reflects her psychological status expressed in different hormones that passes to the neonate via the milk. One must also remember, human neonates are able to experience stress and produce a variety of stress hormones, namely glucocorticoids, as a result of HPA axis activation. These stress hormones, whether maternally driven or produced by the neonate, are able to modulate the immune response.

The exposure to glucocorticoids, maternally derived or generated in response to stress, hinders the generation of an effective repertoire of memory cytotoxic T cells. This is often a point not addressed from an immunization standpoint. Neonates are heavily immunized during the first 12 months of life. The expected outcome of such immunizations is a lifetime protection against pathogens they are being immunized against. However, evidence presented in this chapter clearly demonstrates that such
on assumption warrants further investigation. The data presented in this chapter clearly demonstrate a hindered CTLm repertoire generated under conditions of stress-like glucocorticoids levels. Therefore, one could safely hypothesize that immunity to vaccinations is also being generated at a much reduced level than originally thought. Several studies support this notion, it has been well documented that medical students immunized against Hepatitis during “stressful” periods of their medical education resulted in a dampened immune response (Glaser et al., 1992). More specifically, the memory T cell proliferative response to several early and late Epstein-Barr virus (EBV) polypeptides was significantly decreased during these “stressful” periods (Glaser et al., 1993). Despite such intriguing evidence, there has been a lack of studies examining the role of glucocorticoids, maternally driven or generated in response to stress, on the neonate’s ability to mount a lifelong protection in response to vaccinations received early in life.
Chapter V: Other findings in support of the hypothesis

Several experiments were performed to further elucidate the impact of maternal psychological stress on neonatal T cell adaptive immune response. The findings described herein were profound and present a need for reevaluating several clinical practices for both mother and neonate.

A. Effects of stress-like levels of glucocorticoid on the neonate body weight

The administration of glucocorticoids to the mothers, and their subsequent transfer to the neonates, reduced the neonate weight gain (Figure 33A). This effect was temporary, as 10-14 days after removal of corticosterone-supplemented water, the body weight of neonates who nursed from mothers with access to corticosterone-supplemented water was indistinguishable from their vehicle counterparts. These effects were not attributed to inadequate maternal care or malnourishment as maternal care (collecting all the pups in the nest, grooming of pups, and frequent feedings) was similar to that demonstrated by mothers exposed to vehicle-supplemented water. Additionally, no differences were observed in the neonatal serum glucose levels between pups exposed to stress-like levels of glucocorticoids and their vehicle counterparts. This reduction in neonatal total body weight was mediated via stress hormone-mediated mechanisms, namely corticosterone and epinephrine, as exposure of neonates to stress-like levels of glucocorticoids and a glucocorticoid receptor antagonist restored neonatal body weight to
the levels observed in neonates who were exposed to vehicle and a glucocorticoid receptor antagonist (exposure to a glucocorticoid receptor antagonist did not impact neonatal body weight); (Figure 33B). Moreover, neonatal exposure to stress-like levels of glucocorticoids and a β adrenergic receptor antagonist nadolol (NAD) restored the body weight of these neonates to levels observed in neonates exposed to vehicle and a β adrenergic receptor antagonist (Figure 33B). These findings further confirm a role for both HPA axis and sympathetic nervous system hormones in regulating neonatal body weight. Interestingly, maternal body weight followed the same pattern; the weight gain for mothers exposed to stress-like levels of glucocorticoid lagged behind their vehicle counterparts, and these effects were reversed with the removal of corticosterone or the administration of a glucocorticoid receptor antagonist.
Figure 33

A.

Pups Weight (g) After 6 Days of Treatment

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Vehicle</th>
<th>Corticosterone</th>
<th>Epinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g) at 12 Days of Age</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>Veh</td>
<td>Cort</td>
<td>Cort + RU486</td>
<td>Cort + Nad</td>
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<td>3</td>
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<tr>
<td>5.5</td>
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</tbody>
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B.

Pups weight in (g) at 12 Days of Age

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>Cort</th>
<th>Cort + RU486</th>
<th>Cort + Nad</th>
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<tbody>
<tr>
<td>Weight (g)</td>
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<tr>
<td>0</td>
<td>Veh</td>
<td>Cort</td>
<td>Cort + RU486</td>
<td>Cort + Nad</td>
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<td>5.5</td>
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Figure 33

Beginning 6 days postpartum and continuing for six consecutive days mothers were provided with either corticosterone-, epinephrine-, or vehicle-supplemented water, changed every other day. In the experiments involving receptor antagonist, daily s.c. injections of the glucocorticoid receptor antagonist RU486 (25 mg/kg) or β adrenergic receptor antagonist Nad commenced one day prior to the start of corticosterone- or vehicle-supplemented water and continued for seven days. Mothers and neonates were weighed daily; results represent body weight ± SEM.
B. CD8$^+$ T cell depletion and neonatal health in the context of stress-like levels of glucocorticoid and HSV-infection

Stress-like levels of glucocorticoids, maternally derived or administered directly to the neonate, severely increased HSV-associated mortality, as reported earlier. We speculated that this increased mortality was attributed to the impaired CD8$^+$ T lymphocyte function, also reported elsewhere. Therefore, we sought to determine the impact of neonatal CD8$^+$ T lymphocyte depletion on HSV-associated mortality. To accomplish this task, we injected monoclonal anti-CD8$^+$ antibodies. As expected, these injections depleted the CD8$^+$ T lymphocyte repertoire from these neonates (Figure 34A). Having demonstrated that CD8$^+$ T lymphocyte depletion is possible, we exposed neonates, after the depletion of their CD8$^+$ T lymphocyte repertoire, to stress-like levels of glucocorticoid for 6 consecutive days. On the morning for the sixth day, neonates were infected with HSV-1 in the rear footpads and monitored for HSV-associated mortality. To our surprise, the survival of neonates, in the absence of CD8$^+$ T lymphocyte and presence of stress-like levels of glucocorticoid, was greater than the vehicle counterparts whose CD8$^+$ T lymphocyte repertoire was depleted (Figure 34B). We hypothesized that neonates who were exposed to stress-like levels of glucocorticoids, the depletion of CD8$^+$ T lymphocytes prevented the HSV-associated brainstem edema as reported earlier. This hypothesis is supported by a report describing how psychological stress enhances innate immunity while impairing cellular immunity (Paik et al., 2000). This observation could provide an explanation for our peculiar finding, as the presence of glucocorticoid may have enhanced the innate immune response and aided in the clearance of infection, at the same time, the depletion of CD8$^+$ T lymphocyte prevented the HSV-
associated brainstem pathology reported earlier. Additionally, the absence of elevated glucocorticoids (vehicle) may have prevented the suggested enhancement of the innate immune system and in the absence of the CD8$^+$ T cell repertoire HSV-infected neonates suffered increased HSV-associated mortality. One must also keep in mind that these anti-CD8 antibodies are not specific for T lymphocytes, they also deplete all the cells displaying the CD8 receptor on their surface. One cell type that comes to mind are dendritic cells. The depletion of these cells in itself has detrimental effects on T cell activation as is discussed elsewhere.
Figure 34

A. Percentage Cumulative Survival vs. Days Post Infection for different treatment groups: Veh, Cort, Anti CD8 Veh, and Anti CD8 Cort. Significant differences are indicated with p-values.

B. Percentage of Splenic-Derived T Cells post infection: HSV Anti CD8, Anti CD8, and Naïve. Significant differences are indicated with p-values.
Anti-CD8 antibodies were injected peritoneally beginning immediately post-delivery. At 6 days of age, corticosterone- or vehicle-supplemented water was made available to mothers for six consecutive days. At 12 days of age, neonates were infected with HSV-1 in the rear footpads and monitored for up to 28 days for mortality. As previously noted, the survival of pups that nursed from mothers with access to corticosterone supplemented tap water was 31%, which is significantly lower than their vehicle counterparts (84%). However, depleting CD8$^+$ cells prior to infecting the neonates improved the survival for the corticosterone treated pups to 74% while reducing the survival of their vehicle counterparts to 48%. Values represent mean ± SEM.
C. The administration of epinephrine to the mothers and its impact on neonatal health

To further elucidate the effects of maternal psychological stress, and bearing in mind that stress hormones extend beyond glucocorticoids to the activation of the sympathetic nervous system and the increased synthesis and release of catecholamines, we made available to nursing mothers drinking water supplemented with epinephrine. First, we demonstrated using MS/MS/LC that maternally derived epinephrine is transferred from mother to neonate via breast milk. Having established that this transfer takes place, we began to examine the neonates for stress-hormone related effects. When examine \textit{ex vivo}, splenic cellularity for neonates who nursed from mothers with access to epinephrine-supplemented water was significantly lower than their vehicle counterparts (Figure 35). A similar reduction was observed when corticosterone-supplemented water was used (Figure 7). This observation further confirms a role for sympathetic nervous system-derived hormones in the regulation of splenic cellularity, the major secondary lymphoid organ reflecting the status of other lymphoid organs. Similar to the partial restoration in splenic cellularity observed when a glucocorticoid receptor antagonist was administered (Figure 17), the administration of a \(\beta\) adrenergic receptor antagonist only partially restored splenic cellularity (data not shown). This further confirms that splenic cellularity is controlled via glucocorticoid and sympathetic nervous system mediated mechanisms.
Figure 35

Cellularity

Naive  Vehicle  Corticosterone  Epinephrine

$p < 0.05$
Figure 35

Six days postpartum, epinephrine-supplemented water was made available to nursing mothers for six consecutive days. The water was changed daily and was provided in bottles shielded from exposure to light. On the morning of the 6th day, spleens were harvested from mothers and neonates. Spleens were homogenized and cellularity was determined using trypan blue dye exclusion. Values represent mean ± SEM.
D. Maternally derived CTL are not transferred to the neonate via the milk

In humans, maternally derived lymphocytes are passed to the neonate via the milk. This is evident by the presence of pathogen-specific CTL in neonates as young as few days old without having encountered that pathogen before (Hanson et al., 2003). Therefore, it was logical to hypothesize that in C57BL/6 mice maternal lymphocytes may also be transferred from mother to neonate via the milk. To test this hypothesis, we infected mothers with HSV-1 in the footpads six days postpartum. Five days post-infection, we harvested the spleens of neonates and examined the homogenates using flow cytometry analysis. We were not able to detect any HSV-specific T lymphocytes in the neonates (Figure 36), despite the presence of these cells in the nursing mother. This finding emphasizes the limitations in using animal research models. On the other hand, the absence of maternally derived pathogen-specific T lymphocytes, and the presence of profound effects of maternally derived glucocorticoid on neonatal HSV-specific CTL described elsewhere clearly demonstrates that the increased HSV-associated morbidity and mortality are indeed attributed to glucocorticoid-mediated mechanisms in the neonate and their T cells.
Figure 36

% of Neonatal CD8^+gB_{598-585}^+ Cells

- Naive
- HSV

Background
Figure 36

On day six postpartum, mothers were infected with HSV-1 in the rear footpads. Five days post-infection, at the peak of CTL response, spleens from mothers and neonates were harvested and processed. Single-cell suspensions were prepared and stained using PE-conjugated gB_{498-505} tetramer. The average CD8^+ gB_{498-505}^+ T lymphocytes from neonates that nursed from non-infected mothers are represented with the filled bar, while the average from neonates that nursed from HSV-infected mothers is represented in the non-filled bar. Values represent mean ± SEM.
Chapter VI: Summary, Discussion, and Future Directions

A. Summary

Evidence presented herein begins to shed light on the effects of maternal-neonatal interactions on the role of maternally derived stress-like levels of glucocorticoids and catecholamines on the neonatal T cell adaptive immune response. Reduction of glucocorticoid-mediated mechanisms were demonstrated to be responsible for the increased HSV-associated morbidity and mortality. Glucocorticoid-mediated mechanisms reduced the expression level of IL-2Rα on the surface of HSV-specific CTL. Additionally, these maternally derived stress hormones also reduced the levels of IL-2 produced by these cells. These two events, and possibly others, culminated in the reduced proliferative ability of splenic-derived CTL. This reduction in HSV-specific CTL was evident by the reduced number and percentages of CD8⁺gB₄₉₈-₅₀₅⁺ cells in the popliteal lymph nodes of HSV-infected neonates who nursed from mothers with access to corticosterone-supplemented water. Additionally, when popliteal lymph node-derived lymphocytes were stimulated \textit{ex vivo} using gB₄₉₈-₅₀₅ peptide, these lymphocytes produced less IFN-γ. More importantly, when these popliteal lymph node-derived T cells were assessed for their ability to lyse HSV-infected cells, a severe hindrance was exhibited in the cells derived from neonates exposed to maternally derived stress-like levels of glucocorticoids.

The effects described extended beyond the primary immune response against HSV infection to affect the memory CTL repertoire (CTLm). Even though stress-like levels of maternally derived glucocorticoids did not target a specific CTLm population
per se, it did, significantly reduce the number of HSV-specific effector CTLm in the spleens of mice who were exposed to these stress-like levels as neonates. Additionally, the functional capacity of these memory CTL was impaired when the cells were stimulated \textit{ex vivo}, as was evident by the reduced percentage of CTLm IFN-\(\gamma^+\), and reduced percentage of cells that are able to lyse HSV-infected cells.

The impairment of the primary immune and memory immune response to HSV infection was mediated via type II glucocorticoid receptor mechanisms. The administration of a glucocorticoid receptor antagonist reversed the HSV-associated morbidity and mortality. This restored survival was the reflection of a restored neonatal HSV-specific CTL number and function.

Lastly, neonatal exposure to maternally derived sympathetic nervous system-derived hormones, namely epinephrine, resulted in effects similar to those observed when neonates were exposed to stress-like levels of glucocorticoids. These findings support the hypothesis that psychological stress activates both the HPA axis and the sympathetic nervous system resulting in immunomodulation in both the primary and memory CTL repertoires generated in response to HSV-1 infection.
B. Discussion

Evidence presented herein warrants further review for the management of mothers and neonates alike. Neonatal exposure to stress-like levels of maternally derived stress hormones resulted in increased HSV-associated morbidity and mortality via type II glucocorticoid receptor, and β adrenergic receptor mediated mechanisms. These stress hormones are the end results of the host response to psychological stress. This response to stress involves the activation of the HPA axis and the sympathetic nervous system in an attempt to restore homeostasis. As demonstrated elsewhere, stress-like levels of these hormones impaired the neonatal T cell-mediated immunity generated against HSV-1 infection. The maternal neonatal interaction is necessary for both mother and neonate. The calming effect of breastfeeding and maternal care reduces the stress levels for the mothers and with obvious benefits for the neonates.

Postpartum depression is a leading complication in childbirth and one of the leading stressors for mothers post delivery (Gaynes et al., 2005). Approximately 20% of women experience major or minor postpartum depression symptoms and, surprisingly, only 20% of those women are ever detected and managed (Gaynes et al., 2005). Mothers experiencing postpartum depression are more likely to have attachment issues with their infants, further fueling the postpartum depression cycle (McMahon et al., 2006). These attachment issues do not only worsen the postpartum depression cycle for the mother, but also affect the neonate as demonstrated in disrupted infant sleep, feeding issues, temper tantrums and, not surprisingly, childhood depression later in life when these children were followed in a 13-year longitudinal study (Halligan et al., 2007). Despite the plethora of publications addressing postpartum depression, there is a lack of studies
examining the impact of elevated maternal stress hormones on neonatal immune response to infectious pathogens. The evidence presented herein demonstrates that neonatal exposure to elevated levels of stress hormones negatively impacts the neonate T cell adaptive immune response against HSV-1 infection. This impairment extends beyond the primary response to also hinder the memory T cell repertoire with potentially grave implications. The suboptimal primary immune response, as a result of exposure to elevated levels of stress hormones, makes the neonate susceptible to increased pathogen-associated morbidity and mortality. Additionally, childhood vaccinations administered while the neonate is subjected to elevated levels of these stress hormones results in an impaired T cell memory immune response, increasing the risk of secondary infection. The perception of an adequate/protective acquired immunity in response to childhood vaccinations must be re-visited. While these vaccinations are critical, the assumption of protection must be verified with antibody titer testing or, at least, the administration of a booster immunization. Potential employees in healthcare settings, where exposure to body fluids is possible, are tested for the presence of hepatitis B virus-specific antibodies, a vaccine usually received during the first few months of life. If the hepatitis B virus antibody titer was determined to be below the protective level, a booster vaccination should be administered.

The findings presented herein begin to shed the light on the short- and long-term implications of maternal stress on neonatal health and wellbeing. Further investigation into the cause and treatment of maternal postpartum depression will certainly benefit both the mother and the neonate. Additionally, childhood vaccination strategies and timing is an issue that deserve further evaluation. Not only should the neonatal physical attributes
such as height/weight, temperature, and growth should be documented, but also maternal stress indices available through a variety of stress evaluation tools should be documented.
C. Future Directions

Research delineating the impact of stress-like levels of various endocrine products on neonatal immune response to infectious pathogens is an area that must be further investigated. The currently-available T cell-glucocorticoid receptor (GR)-knockout mice could be used to further elucidate the impact of elevated levels of stress hormones on neonatal T cell mediate immunity. The use of these mice enable us to study the impact of glucocorticoid type II receptor signaling on modulating the T cell-mediated immune response in the neonate. Having the GR conditionally knocked out in the T lymphocyte lineage, with adequate expression in other cells and tissues, enables us to isolate the effects of glucocorticoids on different cellular compartments. Using this knock put mouse model we will be able to determine to what extent stress hormones impairment of the CD8^+ T lymphocytes (described herein) contributes to the increased morbidity and mortality (also described herein)? Additionally, using this mouse model will enable us to closely examine the impact of glucocorticoids on CD8^+ T lymphocyte internal processes such as T cell receptor signaling, gene transcription and translation of various pro-inflammatory cytokines, and intra-cellular trafficking of lysosomes and the release of lysosomal contents. This in depth understanding of the immunomodulatory mechanisms affected by elevated glucocorticoids elicited by psychological stress will assist in translating the findings described herein from bench to bedside.
Proposed Model

Elevated Levels of Stress Hormones

- Via receptor-mediated mechanisms:
  - Decreased production of IL-2 and expression of IL2Rα
  - Decreased T cell proliferation

Decreased Splenic Cellularity

- Decreased number and percentage of HSV-specific CTL
- Impaired pro-inflammatory cytokine production by HSV-specific CTL
- Impaired HSV-specific CTL lytic activity
- Impaired HSV-specific CTLm repertoire

Increased HSV-associated Morbidity and Mortality

- Increased brainstem edema, lymphocytic perivascular cuffing and neuromophagia
- Increased the incidence of herpes simplex encephalitis
References


acute exposure to high cortisol levels is sufficient to induce the enzyme. J Neurochem 58, 1853-1862.


Burchett, S., Mohan, K, Corey, L, Wilson, CB, 1987. Delayed production of interferon-gamma (IFN gamma) and tumor necrosis factor (TNFa) by mononuclear cells (MC) of herpes simplex virus (HSV) infected neonates (NB). Pediatr Res 21, 309A.


Gaillard, R.C., Riondel, A., Muller, A.F., Herrmann, W., Baulieu, E.E., 1984. RU 486: a steroid with antiglucocorticosteroid activity that only disinhibits the human pituitary-adrenal system at a specific time of day. Proceedings of the National Academy of Sciences of the United States of America 81, 3879-3882.


Gessani, S., McCandless, S., Baglioni, C., 1988. The glucocorticoid dexamethasone inhibits synthesis of interferon by decreasing the level of its mRNA. The Journal of biological chemistry 263, 7454-7457.


Nair, A., Hunzeker, J., Bonneau, R.H., 2007a. Modulation of microglia and CD8(+) T cell activation during the development of stress-induced herpes simplex virus type-1 encephalitis.


Selye, H., 1941b. Variations in organ size caused by chronic treatment with adrenal cortical compounds: An example of a dissociated adaptation to a hormone 76, 94-99.


Curriculum Vitae

Education

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1998-2001 University of Maryland  College Park, MD
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