Chronic activation of corticotropin-releasing hormone pathways impairs B cell development and thymus-dependent humoral immune responses

by

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

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Preface

I have prepared my dissertation in accordance with the guidelines set forth by the Graduate Program of the School of Medicine, Oregon Health and Science University. This manuscript consists of a general introduction, three chapters of original data, and a section with summary/general conclusions. The references cited for all chapters are listed together at the end of the text and follow the format of The Journal of Immunology.

Chapter two contains data, figures and text as they appear in the original paper published in the Journal of Immunology (1). Chapter three is a manuscript that has been prepared for publication and submitted to The Journal of Immunology. Chapter four represents a manuscript in preparation for submission.

Abstract

Corticotropin-releasing hormone (CRH) is a central mediator in the response to stress. This peptide hormone is produced predominantly in stress-responsive sites in the brain and regulates behavioral, sympathetic nervous system, and neuroendocrine activation during stress. The neuroendocrine response to stress consists of activation of the hypothalamic-pituitary-adrenal (HPA) axis and is initiated when neurons of the paraventricular nucleus of the hypothalamus release CRH in response to novel or adverse stimuli. CRH then activates production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn causes glucocorticoid release from the adrenals.

Stress is well known to affect the immune system, and glucocorticoids have been shown to mediate many immune effects of stress. We have developed a genetic model of chronic CRH overexpression, CRH transgenic (CRH-tg) mice. These mice express high levels of CRH in areas of the brain and periphery that normally express CRH or that upregulate CRH in response to stress. A hallmark of these mice is continuous activation of the HPA axis, wherein circulating corticosterone levels are similar to levels seen in WT mice following acute stress. In addition, behavioral and physiologic features of these mice mimic effects of stress, and this phenotype persists for the life of the animal. Thus, these mice provide a useful model in which to examine the immune consequences of chronic activation of CRH-responsive pathways.

I was interested in the effect of chronic activation of CRH-responsive pathways on humoral immunity. Exogenous glucocorticoids diminish thymus-dependent antibody responses, and acute stress often diminishes humoral responses as well, although these findings are somewhat

inconsistent. However, most studies have been done using acute hormone administration or acute stress. Thus, I used CRH-tg mice in order to test the hypothesis that chronic activation of CRH pathways diminishes humoral responses.

I found that chronic CRH expression does inhibit antibody responses to thymus-dependent antigens and that this effect is regulated at multiple levels. First, CRH overexpression alters B lymphopoeisis, selectively depleting lymphocytes at the pre B cell stage of development. In addition, CRH-tg mice mount a humoral response that differs both quantitatively and *qualitatively* from WT mice. The qualitative changes include diminished IgG, altered antigen specificity, and poor memory. The pattern of these changes suggested a common underlying mechanism, namely dysfunction of germinal centers. Thus, I hypothesized that CRH overexpression impaired germinal center formation upon immune challenge.

I tested the ability of CRH-tg and WT mice to mount a germinal center response upon immunization with thymus-dependent antigens and found a near complete absence of germinal centers in CRH-tg mice. Similarly, treatment of WT mice with exogenous corticosterone inhibited germinal center formation, indicating that elevated glucocorticoids in adults are sufficient to mediate this effect. While the mechanism of this immune suppression is not completely resolved, evidence indicates that chronic elevations in corticosterone impairs germinal center formation, in part, via disturbing the microenvironment required to support this immune response.

Chapter 1—Introduction

The broad goal of my research is to understand how activation of the stress response affects the immune system. In particular, I am interested in how chronic activation of CRH-responsive pathways affects the development and maturation of humoral immunity. To appreciate interactions between these systems, it is important to understand 1) the physiology of CRH and related neuropeptides in stress-responsive pathways, 2) mechanisms by which CRH-activated pathways interface with components of the immune system, and 3) known effects of CRH on inflammation and adaptive immunity. Figure 1 is a schematic overview of stress-responsive pathways and their interactions with the immune system (see page 27). I wish to emphasize that acute and chronic CRH overdrive often affect immunity very differently, which underscores the importance of our unique genetic model of chronic stress. In the interest of brevity, I provide background on aspects of B cell development and humoral immunity that provide potential targets for CRH-mediated immunomodulation.

1. Corticotropin-releasing hormone (CRH) pathways and the stress response

1.1 CRH and the HPA axis

CRH is a 41 amino acid neuropeptide involved in several stress-responsive pathways, but it was first discovered (and hence named) as the primary stimulator of adrenocorticotropic hormone (ACTH) release from the pituitary, thus initiating the neuroendocrine response to stress (2). Activation of this pathway, the hypothalamic-pituitary-adrenal (HPA) axis, occurs when CRH produced by neurons of the PVN travels the hypophysial portal system and binds a G protein coupled receptor, CRH receptor type I (CRH-R1) on pituitary corticotrophs. This induces ACTH secretion into the bloodstream, which in turn drives release of glucocorticoids (cortisol in humans,

corticosterone in rodents) from the adrenal cortex. Glucocorticoids regulate numerous physiological pathways, allowing an organism to respond to stress, prepare for future or ongoing stress, and return to homeostasis following stress. Foremost among these effects are 1) increased metabolism to meet increased energy demands, 2) decreased reproductive behavior and physiology to conserve resources, 3) inhibition of inflammation to prevent immune activation overshoot, and 4) negative feedback regulation of the HPA axis itself to rein in the neuroendocrine response to stress (3). HPA activation is clearly dependent on both CRH-R1 and CRH because CRH and CRH-R1 knock-out mice do not mount an HPA response to stress (4-6), although some immunologic stressors can activate the pituitary directly. Glucocorticoids released during HPA activation are key mediators of stress-induced immunomodulation and appear to be critical to the effects of chronic CRH overdrive on humoral immune responses as shown in chapter three.

1.2 CRH and sympathetic nervous system activation

CRH is thought to act within the CNS to mediate behavioral and sympathetic nervous system (SNS) responses to stress independent of HPA activation (7, 8). CRH and CRH-R1 distribution is consistent with this hypothesis with expression in limbic areas, which are involved in emotion and stress responses (amygdala, hippocampus, olfactory bulb) and in brain stem regions that regulate autonomic responses (nucleus of the solitary tract, locus ceruleus) (9). Pharmacologic evidence further suggests that centrally-produced CRH plays a key role in stress-induced autonomic activation. Catecholamines, primarily norepinephrine (NE), mediate SNS effects upon release from peripheral nerves originating in the locus ceruleus (LC) in the brain stem. CRH immunoreactive afferent fibers project to structures of the LC that directly activate autonomic fibers, and CRH injected directly into the LC activates sympathetic outflow. Likewise, CRH receptor antagonists injected into the LC prevent sympathetic activation induced by many types of

stress (10). In addition, the PVN-CRH and LC-NE systems positively regulate one another such that hypothalamic CRH activates SNS through projections to the brain stem, and NE fibers that project from the LC to the hypothalamus activate CRH. Such a feed-forward system may be involved in the pathophysiology of depression and anxiety disorders (10). From an immunological standpoint, SNS activation has been implicated, particularly, in effects of *acute* stress or CRH exposure on cellular immunity (11-15). During *chronic* stress or CRH administration, this pathway may play a lesser role in immunomodulation (16, 17), which highlights the idea that acute and chronic CRH overdrive can affect the immune system distinctly.

1.3 CRH and behavioral activation

CRH also appears to mediate stress-induced behavioral changes. CRH injected intracerebroventricularly (icv) induces behavioral effects that resemble closely a state of stress, including freezing, decreased locomotor activity/exploration, decreased food intake, suppressed reproductive behavior, and increased anxiety behavior (10). Moreover, icv injection of CRH receptor antagonists (that block either both known CRH receptors or CRH-R1 only) inhibits the ability of psychological/emotional stress to induce these same behaviors (10). CRH-R1 is critical for the behavioral effects of stress since CRH-R1 antagonists and genetic deletion of CRH-R1 inhibit these responses (4, 5, 18-20). However, peptides related to CRH (see section 2) may also mediate some behavioral effects of stress because CRH KO mice, which cannot mount a normal HPA response to psychological stress, do display behavioral responses to stress (21). It is unclear currently whether this is the case in normal animals or whether other ligands can compensate for CRH in its absence (22). Behavioral effects of CRH are relevant here insofar as our model of chronic CRH overdrive, CRH-tg mice, exhibits many of the well-characterized behavioral changes

observed during stress. This complements physiological data to further support that these mice represent an accurate model of chronic stress (see section 4.2).

2. Additional CRH receptors and related ligands

CRH binds a second G protein coupled receptor, CRH-R2, with an affinity ~10-15 fold lower than CRH-R1. In addition, CRH binds with high affinity to a soluble binding protein, CRH-BP, which is thought to act as a regulatory "sink" for excess peptide (23-25). While CRH-R1 and CRH-R2 are both highly expressed in the central nervous system (CNS), their distribution is distinct. CRH-R1 is most abundant in the paraventricular nucleus of the hypothalamus (PVN) as well as the cerebral cortex, olfactory bulb, hippocampus, septum, amygdala, cerebellum, and brain stem, and peripherally, in the ovary and skin. CRH-R2 is expressed as three distinct functional subtypes (CRH-R2α, CRH-R2β, and CRH-R2γ, with CRH-R2γ expressed in humans but not in rodents) that differ in their N termini due to alternative splicing (26, 27). In the rodent, CRH-R2α is expressed exclusively in the brain, but in areas distinct from CRH-R1, including lateral septum, ventromedial nucleus of the hypothalamus, BNST, dorsal raphe, and the amygdala (28). In contrast, CRH-R2β is found predominantly in the periphery, most notably the heart, skeletal muscle, lung, kidney, intestine, and in non-neuronal areas of the brain, such as the choroid plexus and cerebral arterioles (28-30).

Three other mammalian peptides related to CRH have been cloned recently: urocortin (Ucn), Ucn II, and Ucn III (31-34). Ucn was originally identified in mammals by homology with the fish peptide, urotensin, which is involved in osmotic regulation. Subsequently, Ucn II and Ucn III (in humans also referred to as stresscopin-related peptide and stresscopin, respectively) were discovered based on sequence similarity with Ucn. Ucn binds CRH-R1 with slightly greater

affinity than does CRH, but it binds CRH-R2 with an affinity ~20 fold higher than CRH (34, 35). In contrast, Ucn II and Ucn III are selective agonists for CRH-R2 and do not stimulate CRH-R1. Ucn is expressed centrally in the lateral superior olive and the Edinger Westphal and peripherally in the GI tract, testes, and immune organs (spleen and thymus) (36). Ucn II expression has been detected thus far in the hypothalamus and brainstem (examination of peripheral tissue for Ucn II expression has not been reported) (33), while Ucn III is expressed in areas of the rodent brain including hypothalamus, BNST, brainstem, and amygdala, and, peripherally, in the small intestine and skin (31). In the rat forebrain Ucn III immunoreactive projections overlap with CRH-R2 expression to a much greater extent than does CRH or Ucn, suggesting that Ucn III may be an important physiological ligand for CRH-R2 in these sites (37). Neither Ucn II nor Ucn III has been ascribed a physiologic role to date; however, we and others have generated three independent CRH-R2 knock-out lines, and the phenotype of these mice indicates that CRH-R2 ligands may possess anxiolytic properties that may be important in recovery from stress (38-40). Furthermore, Ucn deficient mice exhibit increased anxiety in the elevated plus maze and open field (41), suggesting that this ligand may mediate anxiolytic properties of CRH-R2, opposing the anxiogenic role of CRH/CRH-R1. Nonetheless, it is important to realize that roles previously attributed to CRH by virtue of studies using exogenous CRH or CRH receptor antagonists or by identifying CRH production with mAb, in some cases, may be fulfilled by a related peptide.

3. Pathophysiology associated with CRH overproduction

In addition to its clear role in stress, CRH is implicated in several disease processes. In humans, major depression, panic disorder, post-traumatic stress disorder and anorexia are all characterized by chronic activation of the HPA axis as shown by elevated CRH in CSF, increased numbers of CRH positive neurons in the PVN, and, excepting PTSD, increased serum cortisol (42-44). While

it is not yet clear whether this is a cause or effect of these diseases, the clinical sequelae of HPA overactivation is clear as these patients suffer from bone density loss, atherosclerosis, and Th1 immunosuppression (44).

Hyposecretion of CRH is implicated in certain diseases as well, including increased susceptibility to autoimmune processes and certain psychological disorders. This is well-characterized in a rat model of streptococcal-cell wall (SCW) induced arthritis, in which a biosynthetic defect in CRH production in Lewis rats results in a blunted HPA response to SCW immunization and subsequent autoimmunity (45-47). Thus, models of CRH dysregulation bear relevance to common disease pathologies.

4. CRH transgenic mice

Stenzel-Poore et al. created a transgenic model of chronic CRH hypersecretion in which the metallothionein promoter drives a rat CRH genomic transgene (48). These mice overproduce CRH in regions of the brain that normally produce CRH or that upregulate CRH upon stimulation (PVN, preoptic area, amygdala, olfactory bulb, lateral septum). Although the metallothionein promoter typically induces widespread transgene expression in the brain and periphery, this did not occur in CRH-tg mice, suggesting that the rat CRH gene contains intronic sequences that regulate its own expression in certain CNS sites and peripheral tissues. In keeping with this, peripheral expression of the CRH transgene follows a pattern similar to endogenous peripheral CRH localization: lung, adrenal, heart, and testis. This restricted CRH transgene expression mimics the physiology of chronic HPA activation and central CRH dysregulation that occurs during stress.

4.1 HPA axis in CRH-tg mice

The hallmark of CRH-tg mice is chronic HPA axis activation. The physical phenotype of these mice mimics Cushing's syndrome: thin skin, hair loss, brittle bones, truncal obesity, and a characteristic buffalo hump (48). These features presumably arise from high circulating corticosterone levels as they resemble effects seen in patients treated with exogenous glucocorticoids and are reversed upon adrenalectomy. In models of chronic physical or psychological stress, animals frequently habituate to the stressor, such that over time, application of the chronic stress no longer induces robust HPA activation. However, responses to a different, superimposed acute stress can still be elicited. Similarly, CRH-tg mice retain the ability to further activate the HPA axis in response to acute restraint stress or endotoxin exposure (see chapter two and unpublished data). The stress-induced rise in corticosterone is delayed in CRH-tg mice, but peak circulating levels are similar to those induced by stress in WT mice and are significantly elevated above the high baseline seen in CRH-tg mice. Thus, despite chronic HPA activation, CRH-tg mice clearly respond to a superimposed stress, again supporting similarities between this model and chronic stress.

4.2 Anxiogenic behaviors in CRH-tg mice

As described above, CRH is considered a critical mediator of stress-induced behaviors independent of HPA activation. Due to high levels of CRH expression throughout the brain, CRH-tg mice were anticipated to exhibit anxiogenic behavior. Indeed, these mice show increased anxiety in the elevated plus maze, light-dark exploration, and responses to novel environments (49-52). Importantly, central administration of the CRH receptor antagonist, α-helCRH9-41 reverses anxiogenic behavior (50). Stress also causes decreased sexual behavior, and pharmacological studies indicate that CRH is involved in this process (53). Likewise, CRH-tg

females show impaired sexual receptivity that is depicted by aggressive behavior towards normal males (51). Additionally, CRH-tg mice have learning and memory deficits, consistent with the proposal that CRH affects learning plasticity (54). Thus, chronic elevations in central CRH are sufficient to increase anxiety and alter learning, similar to behavioral responses to stress. Moreover, CRH-tg mice do not appear to habituate to the effects of CRH despite lifelong elevations in central CRH expression. CRH-tg mice exhibit both neuroendocrine and behavioral features consistent with chronic stress and, thus, provide a model well-suited to investigate immune consequences of elevated, but physiologically relevant, CRH expression.

5. CRH and immune effects

As a CNS peptide hormone and neurotransmitter, CRH can potentially affect immune parameters via three major pathways. Glucocorticoid release through HPA activation is the most well-studied mechanism of immune modulation by CRH and stress. In addition, as described above, CRH modulates SNS activation, which can affect immune parameters via peripheral catecholamines. Finally, peripheral CRH released by peptidergic nerve terminals or produced at inflammatory sites may directly influence cells of the immune system (55-57). In vitro, CRH can exert many direct effects on leukocytes, but the in vivo significance of these studies is unclear since CRH availability and dose at relevant sites is not well-characterized. Here, I focus the discussion on in vivo effects of CRH, elucidated either by central administration of CRH or by antagonizing CRH receptors during psychological or inflammatory stress.

5.1 Central effects of CRH

CRH acting centrally can affect myriad immune parameters. It is important to appreciate that the duration and dose of CRH or the type of stress applied affects what components of the immune

system are altered, in which direction (immunoenhancing or immunosuppressive) these components are altered, and the mechanism by which CRH exerts its effects. Also, in many circumstances immune effects of stress or CRH result from a combination of HPA and SNS activation, with partial effects seen when one pathway is blocked. Most studies have examined effects of CRH on "baseline" immune parameters that are antigen independent, and most of these have examined effects of acute CRH. Despite the aforementioned caveats regarding timing and dosage of CRH/stress, generalizations are outlined below that describe fairly consistent findings among different experimental models.

5.1.1 CRH effects on lymphocyte proliferation and cellular immunity

In general, acute stress or central administration of CRH decreases mitogen-induced lymphocyte proliferation and NK cytotoxic activity in naïve (unimmunized) animals (12, 13, 15, 58-61). Centrally administered CRH receptor antagonists inhibit the effect of acute stress on these immune parameters, further supporting that CRH is a central mediator of such stress-induced immune changes. Most evidence indicates that CRH alters NK activity and lymphocyte proliferation via sympathetic nervous system activation, rather than HPA activation, because β-adrenergic receptor antagonists and chemical sympathectomy, but not adrenalectomy, reversed the stress-induced immune suppression (13, 62, 63). Moreover, CRH injected directly into the LC elicited similar immune changes (64, 65). Long-term (7 days) icv infusion of CRH also inhibited lymphocyte proliferation, but in contrast, adrenalectomy abolished these immune changes (16, 17). This highlights the point that acute stress and chronic CRH activation can alter immunity differently.

5.1.2 CRH and humoral immunity

While stress has been shown in numerous situations to alter antibody responses (see chapter 4), limited studies have specifically investigated the effects of CRH on humoral immunity. Two studies examined the acute effect of icv CRH on the subsequent antibody response to immunization with KLH. CRH administered immediately before immunization significantly decreased IgG titers, but only variably affected IgM (14, 66). Importantly, the dose of immunogen was critical in that CRH affected the humoral response to low doses of antigen (3-30 ug/kg) in the absence of adjuvant, but not to high doses of antigen (300 ug/kg). Thus, acute CRH elevation may affect humoral responses particularly when immune stimulation is limiting. In these studies, sympathetic nervous system activation was implicated in the IgG decrease, but the role of adrenal hormones is difficult to interpret because following adrenalectomy, antibody responses are limited (14, 67). Thus, previous data regarding the influence of elevated CRH on antibody production were limited to acute effects and did not determine the role of elevated corticosterone. I wished to determine whether *chronic* CRH overdrive modulates humoral responses and explore the hormonal and immunological mechanisms by which these changes occurred.

5.2 Direct effects of CRH on inflammation

5.2.1 Peripheral expression of CRH and CRH receptors

CRH expression is most robust in the CNS and rarely detected in the circulation. However, CRH is found peripherally, where it is produced locally in peripheral tissues or released from peripheral nerves. Immunoreactive CRH is present in inflamed tissue (68-74) as well as several normal lymphoid sites such as rat thymus and spleen (75-77), although many of these studies detected CRH with reagents later shown to cross-react with Ucn (57, 78). Both CRH and Ucn mRNA are expressed in lymphoid tissues (36, 75, 79, 80), including the red pulp and the marginal zone of rat

spleen (77), and Ucn mRNA expression in the thymus and spleen is regulated by immune challenge (36). Several types of leukocytes express CRH receptors (e.g. T lymphocytes, neutrophils, monocytes) as do the thymus and spleen (80), and in the case of neutrophils, CRH receptor expression increases upon immunologic or psychological stress (81, 82). Thus, CRH ligands and receptors are located in a pattern suggesting they may directly regulate immune responses as indicated below.

5.2.2 Pro-inflammatory effects of peripheral CRH

Most studies on direct effects of CRH in the immune system have been performed in vitro and demonstrate that CRH stimulates various leukocyte populations to increase proliferation or cytokine production via functionally coupled CRH receptors (83-88). Studies that have shown a physiologic effect of endogenously produced CRH have primarily examined inflammatory processes. In contrast to its action via glucocorticoids, peripheral CRH appears to be pro-inflammatory. Adminstration of CRH anti-serum or CRH receptor antagonist showed that endogenous CRH increases exudate volume and cellular infiltrate in models of aseptic and antigen-specific inflammation (68, 89, 90), enhances pro-inflammatory cytokine production (91), and induces spontaneous mast cell degranulation (92-94). CRH may augment extravasation, in part, via vasodilation and increased vascular permeability, but CRH receptor expression and functional coupling in leukocyte populations indicates they may provide a direct target as well. For instance, CRH induces mast cell degranulation via direct actions on CRH receptors expressed by these cells (92). The precise source of CRH during these inflammatory processes is not clear, but peptidergic nerve fibers are associated closely with mast cells in perivascular spaces and lymphoid tissues (70, 95). Again, it is possible that CRH-related ligands in the periphery, particularly Ucn III which is expressed in the skin, may contribute to these effects since the anti-

serum to CRH used in some studies may cross-react with other CRH receptor ligands, and CRH receptor antagonists do not discriminate between different ligands for the same receptor.

6. Glucocorticoid actions in the immune system

Glucocorticoids exert a wide array of immunomodulatory effects. It is important to note that low baseline levels of glucocorticoids are, in many cases, required for a normal immune response. Thus, while elevated glucocorticoids may suppress particular immune responses, a complete lack of glucocorticoids may also suppress the same immune response, as described above for antibody responses (section 5.1.2) (67, 96). A vast literature documents effects of elevated glucocorticoids on various immune parameters; however, much of this research has been done in vitro or in vivo using synthetic glucocorticoids (ie-dexamethasone, prednisolone) at supraphysiologic concentrations. Here, I focus the discussion on several broad categories of immune alterations observed in vivo with physiologic levels of biologically relevant glucocorticoids.

6.1 Glucocorticoid effects on inflammation

A major role of glucocorticoids is to limit inflammation during an immune response. The pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 produced early in an immune response activate the HPA axis at the level of the pituitary and hypothalamus (97, 98). Glucocorticoids produced in response to HPA activation then inhibit cytokine production from leukocytes (98). In addition, glucocorticoids potently inhibit inflammatory mediators such as histamine, bradykinin, NO, cyclooxygenase, and adhesion and chemotactic factors (3). This negative regulatory loop is crucial during intense inflammatory processes, such as sepsis; without this feedback, overproduction of inflammatory mediators causes fever, vascular leakage due to breaches in activated endothelium, and subsequent organ failure and death (98, 99). This is illustrated clearly by decreased survival of

adrenalectomized animals when challenged with LPS or highly pathogenic viruses (97-100). Thus, linking the HPA axis with immune activation is an adaptive response, designed to control excess inflammation during immune responses. However, inflammatory mediators are important in mediating early innate defense as well as in upregulating costimulation to activate the adaptive response. Thus, *over-activity* of the HPA axis during a non-immune type stress (physical or psychological) can alter how the immune system responds to a subsequent pathogenic insult, potentially enhancing susceptibility to infection (101-105). Since chronic HPA activation is a hallmark of CRH-tg mice, these mice provide a useful tool with which to investigate how ongoing HPA activation affects responses to a superimposed immune challenge.

6.2 Glucocorticoid effects on leukocyte populations

One well-studied immunomodulatory effect of glucocorticoids is lymphocyte death. Selye noted that stress decreased the size of the thymus and showed this was a result of increased adrenal cortex hormones (106). The thymus responds robustly to glucocorticoids, losing up to 90% of its lymphocytes within a few days, but glucocorticoids also decrease the size and cell number of other lymphoid organs including the spleen, lymph nodes, and Peyer's patches (107-109). Glucocorticoids decrease thymic cellularity via apoptosis of T lymphocytes (110), particularly CD4/CD8 double positive thymocytes (111). B lymphocyte precursors are also quite sensitive to glucocorticoid-induced apoptosis, although this was not appreciated until more recently because glucocorticoids do not change total bone marrow cellularity dramatically (109, 112, 113). This is due, in part, to an increase in neutrophil production in response to glucocorticoids, which masks diminished B lymphocyte numbers when total leukocytes are quantitated. This highlights the point that the effects of glucocorticoids are dependent on cell-type. Because glucocorticoids and stress

are known to diminish lymphocyte populations, I hypothesized that chronic CRH overexpression would decrease lymphocyte populations, particularly T and B cells precursors.

In addition to cell survival, glucocorticoids affect lymphocyte populations through cell trafficking. For instance, glucocorticoids increase peripheral neutrophil numbers not only by increasing neutrophil production, but also by expediting their release from the bone marrow (114-118). During short periods of stress, glucocorticoids decrease most populations of circulating leukocytes, but these cells redistribute to the skin and lymphoid organs (spleen, BM, lymph nodes) (117-119). This redistribution is likely adaptive because injury and/or infection often accompany acute stress; thus, mobilization of immune cells to the site of pathogen entry (the skin) and to the sites of lymphocyte activation (peripheral lymphoid organs) prepares the host for possible infection. Moreover, leukocyte redistribution during acute glucocorticoid exposure correlates with enhanced T cell-mediated immune responses in the skin (delayed-type hypersensitivity reaction, DTH) (120, 121). It is important to emphasize that acute and chronic exposure to glucocorticoids often exert distinct immunomodulatory effects, because the same stressor (restraint in this case) applied chronically induced the opposite effect—the DTH response was decreased significantly (122). Thus, I wished to determine whether chronic CRH overproduction and HPA activation would lead to changes in leukocyte populations similar to what has been observed during acute stress or glucocorticoid treatment.

6.3 Glucocorticoid effects on leukocyte function

6.3.1 Cellular immunity

Altered trafficking, as mentioned above, can indirectly impact leukocyte function by changing the microenvironment of a cell. In addition, glucocorticoids can alter cell function directly. For

instance, glucocorticoids shift the balance of a cytokine response from Th1 to Th2 by suppressing IL-12 and IFNγ production while augmenting IL-4, IL-10 and TGFβ. This may contribute to impaired cellular immunity observed during chronic stress wherein glucocorticoids diminish cytolytic function of cytotoxic T lymphocytes and NK cells (102-104). One might predict that a shift from Th1 to Th2 type responses would maintain or even augment humoral immunity. However, exogenous glucocorticoids and stress generally inhibit thymus-dependent antibody responses (see 6.3.2 and chapter 4). Thus, I proposed that a distinct mechanism might account for decreased humoral responses during chronic HPA activation and investigated the effect of chronic HPA activation on germinal center formation and FDC networks.

6.3.2 Humoral immunity

The effects of endogenous stress-associated levels of glucocorticoids on B lymphocyte function are supported by indirect evidence. In many instances, stress has been shown to decrease antibody responses to pathogens or protein antigens in human and animal studies (see chapter 4), and this correlates with increased circulating glucocorticoids. However, the direct role of glucocorticoids in stress-induced immunomodulation of humoral immunity is difficult to establish because humoral responses require baseline levels of glucocorticoids, as described earlier (section 5.1.2). Thus, studies examining glucocorticoid effects on antibody responses have utilized glucocorticoids administered exogenously. Such treatment clearly inhibits antibody responses, with early studies dating back over 50 years. It is important to note that the timing, dose, and type of glucocorticoid, as well as the dose of antigen all contribute to the effect of glucocorticoids on humoral immunity. Berglund demonstrated this clearly in a series of publications which showed that cortisone had the greatest effect on primary antibody responses when it was administered daily for several days prior to through one day following immunization (123). Furthermore,

corticosterone more potently inhibited humoral responses to low antigen doses compared to larger antigen doses (124). Both of these parameters consistently affect the degree of corticosterone-induced specific antibody inhibition (125-127). Subsequent to these early studies, a consistent pattern has emerged in which glucocorticoids diminish IgG titers and secondary or memory responses to a greater extent than primary IgM responses (126, 128-131).

That IgG titers are more sensitive to glucocorticoid inhibition could indicate that glucocorticoids inhibit isotype switching or that, once switched, glucocorticoids preferentially inhibit IgG synthesis. Indirect evidence suggests that both mechanisms may be important. Glucocorticoids administered in the absence of deliberate immunization diminish circulating levels of total IgG more severely than total IgM (132, 133), indicating that ongoing IgG synthesis is impaired (notably, most studies have found only minor effects of glucocorticoids on Ig catabolism; therefore, increased turnover cannot account for decreased IgG (133)). However, when glucocorticoids are administered near the time of immunization, specific IgG production is diminished, and this correlates with a decreased number of IgG-producing plasma cells (126, 129, 130, 134). Moreover, glucocorticoids given when isotype switching initiates (~day 5 of a primary response) inhibit both the rise in IgG titers and the ability to mount a subsequent secondary response (135, 136). Intriguingly, high bolus doses of glucocorticoids at this time affect already formed germinal centers, apparently enhancing apoptosis within these structures (135-137). Combined, these data indicated that glucocorticoids inhibit particularly those facets of a humoral response that depend on germinal centers. This suggested that impaired germinal centers might underlie the poor antibody response I observed during chronic HPA activation.

7. Molecular mechanisms of glucocorticoid action

7.1 Glucocorticoid receptors

Glucocorticoids bind two intracellular receptors, mineralocorticoid (Type I) receptors (MR) and glucocorticoid (Type II) receptors (GR). MR, which are abundant in the pituitary and hippocampus, have a higher affinity for glucocorticoids than do GR and thus are more likely to be occupied at low concentrations of corticosterone. The more ubiquitous GR are expressed at high levels in the periphery, notably the thymus and spleen (138, 139). Thus, at basal limiting levels, glucocorticoids bind primarily MR, which have few immune effects, but at elevated levels, glucocorticoids bind GR, which essentially all leukocytes and immune tissues express to varying degrees (140). Glucocorticoid effects are also regulated by corticosterone binding globulin (CBG), a soluble binding protein that prevents corticosterone, but not synthetic glucocorticoids, from activating MR and GR (141).

GR is a ligand-regulated transcription factor in the nuclear hormone receptor superfamily. Similar to other nuclear hormone receptors, GR has a modular structure consisting of a DNA binding domain, a ligand binding domain, and two transactivation motifs (142). In the absence of ligand, GR is retained in the cytosol in an inactive state associated with several heat shock proteins. Upon ligand binding, GR translocates to the nucleus where it controls the transcription rate of target genes via several mechanisms (143). Receptor homodimers bind to glucocorticoid responsive elements (GRE) in promoters, which activate or repress target gene transcription. GR also regulates transcription via protein-protein interactions that co-activate or repress other transcription factors. In addition, GR regulates gene expression indirectly by inducing transcriptional repressors and competing for transcriptional co-factors. Each of these mechanisms is important in GR-mediated immune modulation.

7.2 Anti-inflammatory effects of glucocorticoids

Glucocorticoids alter many immune responses via direct or indirect inhibition of the pro-inflammatory transcription factors, AP-1 and NF-κB. These transcription factors are responsible for the production of numerous immune mediators, a list which includes pro-inflammatory cytokines, chemokines, and adhesion molecules (143). Furthermore, NF-κB is critical to adaptive immune responses as it is involved in signaling through antigen receptors and in maintaining a lymphoid microenvironment conducive to lymphocyte activation (144-151). GR antagonizes AP-1- and NF-κB-mediated transcription via protein-protein interactions and paired GR and AP-1- or NF-κB-responsive elements in promoters (143). In addition, GR upregulates transcription of the NF-κB repressor protein, IκBα (152, 153). I speculate that glucocorticoids suppress humoral responses, in part, via regulating NF-κB-mediated gene transcription because NF-κB is important in BCR signaling and germinal center formation (144-148, 154-157).

7.3 Apoptotic response to glucocorticoids

Despite intense study, specific GR target genes involved in glucocorticoid-induced apoptosis have yet to be identified. Evidence exists that both repression and induction of gene transcription are important, and in most situations transcription and translation are required (158). Because nearly all cells constitutively express the machinery necessary for apoptosis, a current view regards apoptosis as a default pathway that healthy cells actively repress (159). In this model, glucocorticoids and other apoptotic signals may act by *de-repressing* apoptosis in target cells. Thus, GR-mediated transactivation might inhibit transcription of an apoptotic repressor or induce transcription of a protease that degrades an apoptotic repressor. Possible targets include proteins which increase cytosolic Ca2+, depolarize the plasma membrane, or activate caspaces and

nucleases (158, 159), each of which has been implicated in GR-mediated cell death. Mechanisms of GR-mediated apoptosis may vary depending on cell type. Most of the reviewed literature use thymocytes as a model apoptotic cell, while mechanisms of B cell-mediated apoptosis via glucocorticoids have not been examined to my knowledge. Nonetheless, glucocorticoid-induced apoptosis of B lymphocytes and B lymphocyte precursors follows a phenotypic and kinetic pattern similar to that of T cells, suggesting that similar mechanisms may be involved (109, 112, 113, 160). Thus, I hypothesized that in CRH-tg mice, which have elevated glucocorticoids, the pre B cell population may be preferentially diminished.

8. Humoral immune responses

Humoral immune responses are critical to defend against all classes of infectious pathogens, but inappropriate antibody resposes to self-antigens can also lead to autoimmunity. Stress and pathologies involving CRH dysregulation have long been linked to altered protection against pathogens and autoimmune diseases. Previous studies relied on exogenous administration of CRH or CRH receptor antagonists administered during stress in order to study the role of acute increases in CRH on immune dysfunction. These methods are difficult to adapt to a chronic model of CRH overdrive. In addition, exogenous administration of CRH may not reflect physiologically relevant levels or patterns of CRH expression, and CRH receptor antagonists do not discriminate between different ligands for the same receptor. Therefore, I used CRH-tg mice to study the immunological effect of chronic CRH overdrive in a system where expression is physiologically relevant in terms of expression level and anatomical location. My research questions address how chronic CRH drive impacts humoral immunity through alterations in B cell development and maturation of humoral responses to thymus-dependent antigen. The following section will review specific topics of B cell immunobiology related to B lymphopoeisis and germinal center function.

8.1 B cell development

B lymphocyte development from common lymphoid progenitors occurs primarily in the bone marrow after birth. B cell development proceeds as follows: 1) pre-pro B cells express the Pax-5 transcription factor which is necessary for B cell lineage commitment and inhibition of other lineages, 2) pro B cells activate Rag and TdT and rearrange VH, 3) if the VH rearrangement is productive, large pre B cells express VH in combination with VpreB and λ5 (the pre BCR), 4) small pre B cells downregulate pre BCR, reactivate Rag and TdT, and rearrange VL, 5) immature B cells with membrane BCR are selected or, if autoreactive, undergo apoptosis, anergy, or receptor editing. Each of these stages can also be detected by a distinct phenotype of cell surface markers (161). It is important to note that while B cells bearing strongly autoreactive antigen receptors are subject to negative selection, lower affinity autoreactive B cells and secreted antibody are common, particularly in the B-1 peritoneal B cell subset. Furthermore, recent evidence indicates that at least for some carbohydrate specificities, true positive selection on self-antigen is required to develop these specificities (162). While not directly addressed by my research, I speculate that glucocorticoids may affect B cell selection, skewing the repertoire towards low affinity, polyreactive, "natural" antibody-producing B cells (see discussion).

Two checkpoints of B cell development are associated with a high level of apoptosis (163). The first is during the transition from pro to pre B cells in which the newly rearranged VH must pair with the surrogate light chain (SL) to form a functional complex on the cell surface. If a productive VH is not produced or it cannot pair with SL, the absence of pre BCR signaling through Igα/Igβ causes apoptosis (164). While this signaling event apparently does not require an extracellular ligand, it does require cell surface organization (165). The second checkpoint occurs

when immature B cells express surface BCR. Here, if the newly rearranged VL is not productive or cannot pair with the VH, the absence of signaling via an intact surface BCR again causes apoptosis. In both cases, apoptosis is initiated via caspace activation, which is regulated by counteracting effects of bcl-2 (anti-apoptotic) and bax (pro-apoptotic) (163). Pre and immature B cells show the highest ratios of bax:bcl-2 expression. During these stages, a positive signal generated through productively rearranged antigen receptors allows the cell to survive, in part via bcl-2 induction, and artificially increasing bcl-2 expression can protect pre B cells from apoptosis (160).

In DP thymocytes, bcl-2 overexpression partially protects against glucocorticoid-mediated apoptosis (166). In that study, bcl-2 overexpression also inhibited the ability of glucocorticoids to repress transcription factors in the AP-1 and NF-κB families, which are involved in antigen-receptor signaling. By analogy, in pre B cells glucocorticoids may inhibit upregulation of bcl-2 upon pre BCR signaling, thus preventing the rescue from apoptosis that is normally provided via productively rearranged heavy chain in combination with SL. Due to increased levels of glucocorticoids in CRH-tg mice, I postulated that chronic CRH overdrive would impair B cell development through preferential depletion of the pre B cell population. I found this to be true, wherein numbers of developing B cells in the bone marrow of CRH-tg mice are significantly diminished, and pre B cells are decreased disproportionately (see chapter 2).

8.2 Germinal centers

Immunological memory is a key feature of the adaptive immune response. During a thymus-dependent immune response, clones of high affinity antigen-specific memory cells are generated in both T and B lymphocyte compartments. These cells respond much more quickly and robustly

following a subsequent antigen encounter. In addition, individual B lymphocytes are uniquely able to increase the affinity of their antigen receptors via somatic hypermutation (SHM), or gene conversion in certain species, and can alter the effector function of secreted immunoglobulin via class switch recombination (CSR).

8.2.1 Function of germinal centers

Germinal centers are considered an important microenvironment for CSR, SHM, affinity maturation, and memory cell formation. In the primary immune response activated B cells first interact with T cells at the interface of B and T cell areas in lymphoid organs (167). They then move to the follicle where they proliferate and form oligoclonal germinal centers that are histologically identifiable with peanut agglutinin (PNA, a lectin) (168, 169). Within germinal centers B cells undergo rounds of 1) proliferation, during which centroblasts downregulate surface Ig and mutate V regions, and 2) selection, during which centrocytes re-express newly mutated surface Ig and exit cell cycle, and those clones carrying mutations that provide the highest affinity interaction with antigen survive at the expense of clones with lower relative affinity (169-177). Only these high affinity germinal center B cells differentiate into plasma or memory cells (178, 179). During this process, some B cells also switch isotype (180, 181).

A vast literature spanning the past 30+ years provides evidence that germinal centers are critical to CSR, SHM, affinity maturation, and formation of memory cells. During an immune response, highly mutated V genes are found only in the PNA+ (germinal center) B cell population (173, 176, 182), and disruption of already formed germinal centers in the first week of an immune response inhibits memory B cell formation (135, 136). Most recently, a number of mouse knock-out models that lack both germinal centers and the ability to generate an affinity matured, isotype-switched

memory response have supported the earlier studies (183-187). Germinal centers are not an absolute requirement for these processes because affinity maturation can occur in the absence of germinal centers in ectotherms and in LTα knock-out mice, if immunized with a high dose of antigen coupled to strong pro-inflammatory signals (188, 189). However, in most circumstances germinal centers appear to be critical, providing the optimum microenvironment for SHM and affinity maturation, particularly when antigen and/or innate immune stimulation is limiting. Thus, the severe defects in germinal center formation I observe during chronic HPA activation or glucocorticoid exposure likely underlie the functional impairment in humoral immunity.

8.2.2 Germinal center requirements

A complete understanding of all the signals required for germinal centers is still lacking, but some key cellular and molecular players have been identified. On a cellular level, T cells and follicular dendritic cells (FDC) are critical accessory cells. T cell costimulation via CD28-B7 and CD40L-CD40 interaction provide early B cell help. In addition, CD4+ T cells constitute ~10% of germinal center lymphocytes. It is thought that germinal center T cells provide two important functions by responding to antigen that has been internalized, processed and presented in the context of class II MHC on germinal center B cells (190, 191). First, they may help drive proliferation and isotype switching of the highest affinity B cells within germinal centers. B cells with the highest affinity antigen receptor presumably "win" the competition for limiting antigen, which they then can process and present to helper T cells. Secondly, germinal center T cells may provide tolerizing signals to B cells that have mutated their antigen receptor towards self-reactive specificities, thus preventing autoimmunity. A few small, short-lived germinal centers can be formed either in response to TI antigens or in mice lacking T cells (192, 193); however, T cells are necessary to

generate full germinal center responses with concomitant affinity maturation and memory cell formation (194-198).

FDC are non-hematopoeitically derived cells that form an antigen trapping reticulum by means of complement and Fc receptors (191, 199-202). FDC have long been considered critical to germinal center reactions, and studies utilizing TNF-family cytokine/cytokine receptor knock-out mice support this contention. An excellent correlation exists between mice that lack FDC and those that lack germinal centers (183, 184, 186, 187). Furthermore, in instances where FDC can be restored via adoptive transfer of WT B cells or exogenous administration of the missing cytokine, restoration of FDC networks almost invariably re-establishes the capacity to form germinal centers (184, 203-205).

The most immediately obvious role of FDC is trapping and long-term (up to years) retention of antigen in the form of immune complexes (206-209). This may serve several functions. First, during a *secondary* response, antigen is quickly complexed with pre-formed circulating antibody and trapped by FDC, allowing very rapid stimulation of memory B cells and new germinal center formation (210). In addition, Tew and Szakal proposed that antigen retained on FDC regulates the long-term maintenance of antibody titers (209). While antibody titers remain high, the antigen is "hidden" by specific antibody covering exposed epitopes; when antibody titers fall, newly exposed epitopes can then stimulate further antibody production. Finally, later in a primary response (after antibody production has started), antigen trapped by FDC has been proposed to be the source of antigen that drives selection of high affinity somatic mutants (210).

Initiation of primary germinal centers may not depend on antigen trapped by FDC since this typically does not occur until after primary germinal centers have begun to form (211-215). The later expansion phase and full generation of affinity maturation and memory is thought to depend on FDC-trapped antigen-antibody complexes (216) because in the absence of complex trapping, FDC do not support full germinal center or memory responses (217-220). However, this interpretation is complicated by a recent study that showed normal germinal center formation, selection, and affinity maturation after primary immunization in mice lacking detectable immune complex deposition due to a mutation preventing antibody secretion (but allowing normal surface Ig expression and normal B cell development) (221). These results, along with in vitro experiments that show FDC provide (as yet ill-defined) antigen-independent chemotactic, adhesive, proliferative, survival, and activating signals to B cells (210, 222-224), indicate that during primary immune responses, FDC may play critical roles in germinal center reactions independent of antigen-trapping. While their precise roles in germinal centers may be more complex than previously appreciated, FDC are considered critical to the germinal center microenvironment. Their abnormal phenotype in CRH-tg and glucocorticoid-treated mice suggests that this disturbance contributes to the germinal center defect in these mice.

On a molecular basis, recent studies utilizing gene knock-out models have uncovered roles for several pro-inflammatory cytokines and costimulatory molecules in germinal center formation. These include members of the TNF and TNF receptor families—TNFα, LTα, LTα/β, TNF-R1, LTβ-R, CD40, CD40L—knock-outs of which display inhibition or complete failure of germinal center formation (170, 183, 186, 187, 225). As discussed in more detail in chapter three, the cellular targets of some of these molecules are being unraveled. Briefly, TNF and lymphotoxin produced by B cells act on TNF/LT receptors expressed by follicular dendritic cells and their

precursors (184, 185, 203, 204, 226-228). Signaling through these receptors and subsequent activation of NF-κB is required to induce and maintain mature differentiated FDC (145, 148). Thus, these interactions between B cells and stromal cells are critical in creating a microenvironment that can support germinal center reactions and maturation of humoral immune responses. Furthermore, the requirement for NF-κB in maintaining this microenvironment suggests a mechanism by which glucocorticoids may inhibit germinal center formation.

In earlier studies, immunization of CRH-tg mice resulted in a primary response characterized by a 7-fold decrease in PFC compared with WT mice. During the memory response, however, this decrease was exaggerated—CRH-tg mice had 60-fold fewer antigen-specific PFC per spleen (229). In addition, I found that isotype switching to IgG following antigen boost was impaired in CRH-tg mice (see chapter 2), similar to observations in glucocorticoid-treated animals. Such impairments in isotype switching and memory responses suggest impaired germinal center function. Therefore, I hypothesized that chronic CRH overexpression would inhibit germinal centers via elevated glucocorticoid levels.

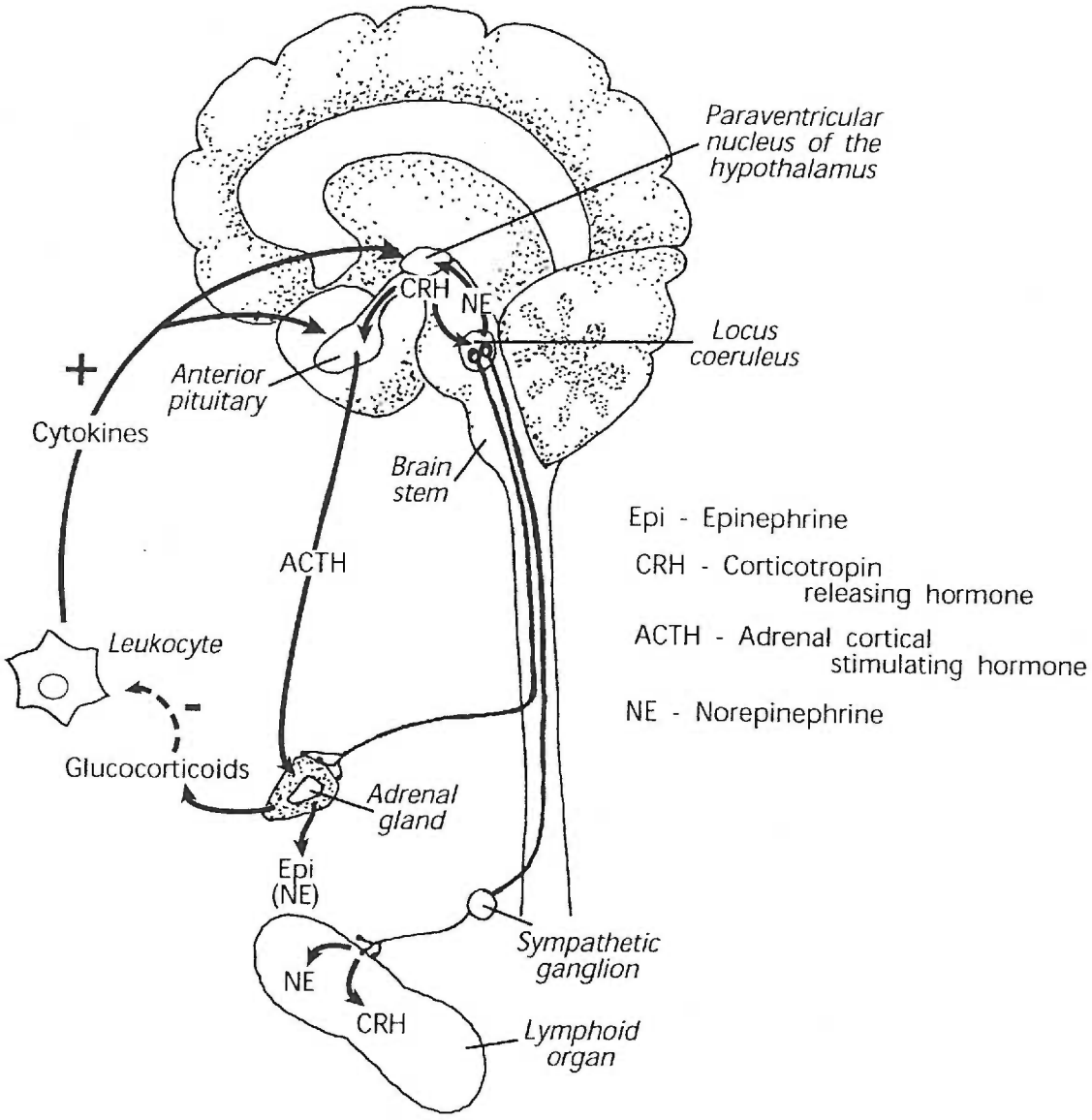

Figure 1. Interactions between CRH pathways and the immune system

Chapter 2—Manuscript #1

A genetic model of stress displays decreased lymphocytes and impaired antibody responses without altered susceptibility to *Streptococcus Pneumoniae*

Running title: Antibody production and host defense during chronic stress

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Abstract

Stress pathways affect immune function, the most notable of these pathways being activation of the hypothalamic-pituitary-adrenal (HPA) axis. Although HPA activation has generally been relegated to an immunosuppressive role, recent evidence suggests that stress and HPA activation can be immunoenhancing in certain situations. In order to investigate specific effects of stress on immune function, we utilized a genetic model of chronic stress wherein transgenic mice overexpress corticotropin-releasing hormone (CRH), a primary mediator of the stress response. In these mice, CRH is overproduced in the brain, leading to chronic activation of the HPA axis. We found that CRH transgenic mice have decreased leukocyte numbers in lymphoid compartments, with preferential loss of B lymphocytes. They also exhibit decreased antibody production and impaired isotype switching in response to immunization with a thymus-dependent antigen, PC-KLH. Despite these deficits, immunization protected CRH-tg and WT mice equally well against lethal challenge with *S. pneumoniae*, an encapsulated Gram positive bacterium known to require antibody-mediated opsonization for clearance. While IgG responses are severely depressed in these mice, IgM titers are only modestly decreased. This fairly robust IgM response may be sufficient to protect against *S. pneumoniae*. Additionally, while total leukocyte numbers are decreased in these mice, neutrophil numbers are increased. This increase in number of neutrophils may compensate for the depressed IgG response, allowing adequate host defense during chronic stress.

Introduction

Stress has long been associated with altered immune function, yet the mechanisms by which this occurs have not been fully elucidated. Several molecules induced during the response to stress have been implicated in immunomodulation, including catecholamines, neuropeptides, and steroid hormones. Corticotropin releasing hormone (CRH) is a central mediator of the stress response. This peptide hormone is produced throughout the central nervous system as well as in several peripheral sites including the pituitary, testes, ovaries, heart, adrenals, and immune tissues (2, 75, 76, 230). CRH released from the hypothalamus initiates the neuroendocrine response to stress by stimulating ACTH secretion from the pituitary. ACTH then drives the release of glucocorticoids from the adrenal cortex. Activation of this neuroendocrine stress pathway, known as the hypothalamic-pituitary-adrenal (HPA) axis, plays a prominent role in the response to psychological, physical or immunological stress (27, 30).

The key hormones of the HPA axis have been shown to modulate immune function. Glucocorticoids have profound effects on immune system development and function (231). While generally considered immunosuppressive due to their ability to induce lymphocyte apoptosis and inhibit pro-inflammatory cytokine production (98), glucocorticoids have recently been shown to possess immunoenhancing properties as well (120, 121). It is also clear that CRH can enhance or diminish immune responses independent of glucocorticoids (73, 85-87, 232-235). Direct effects of CRH in the periphery have been reported (73, 92) and the discovery of CRH and its receptors in immune organs and inflamed joints supports the possibility of paracrine actions for CRH (36, 55, 82) (Stenzel-Poore, unpublished). Thus, it is well recognized that stress mediators can alter specific immune parameters.

However, the effects of chronic stress on immune function are less clear. Our goal is to understand the pathogenesis of immune dysfunction that occurs during prolonged periods of stress. We have generated a *genetic* mouse model of CRH overexpression in order to study the effects of chronic HPA activation on the immune system. These mice (CRH-tg) overexpress CRH in the hypothalamus and other areas of the brain due to the presence of a CRH transgene (48). These animals have increased circulating levels of ACTH and corticosterone (CORT, endogenous murine glucocorticoid), although peripheral levels of CRH are not elevated (48). Importantly, basal levels of ACTH and corticosterone in CRH-tg mice are similar to levels in normal mice during acute stress (Fig.1). CRH-tg mice are capable of HPA axis activation beyond their elevated basal state during a superimposed acute stress—an effect similar to that seen in other models of chronic stress (236, 237). The behavioral profile of CRH-tg mice is also consistent with exposure to chronic stress. CRH-tg mice exhibit increased anxiety, decreased exploration, learning impairment, and decreased reproductive behavior (50, 52). These behaviors exist in CRH-tg animals in the absence of exogenous stress and can be exacerbated further following exposure to stress (50).

Previous studies using these mice reported marked reductions in cell numbers in the spleen and thymus with a more modest reduction in the bone marrow (229, 238). Compared to other cell types, B lymphocytes were found to be preferentially depleted in the spleen and bone marrow and CD4/CD8 double positive T cell precursors were preferentially diminished in the thymus. Antibody responses were also decreased in these mice. Adrenalectomy partially reversed these changes, making it unclear whether glucocorticoids are solely responsible.

These preliminary observations suggested that CRH-tg mice may be quite immunosuppressed. We hypothesized that this immunosuppression may be qualitative as well as quantitative, and that adaptive immunity would be targeted. Therefore, we examined leukocyte populations in primary and secondary lymphoid organs. Because DP T cells were decreased in the thymus, and the bone marrow exhibited a profound loss of B cells, we suspected that certain B lymphocyte subsets may be targeted preferentially. Here, we show developmental alterations of B cell populations in the bone marrow, wherein pre-B cells are depleted preferentially. In investigating lymphocyte function by measuring antibody responses to primary and secondary immunizations, we found that CRH-tg mice display high pre-immune IgM titers and mount robust IgM responses to immunization. However, these animals show poor secondary IgG antibody responses, indicating a predominant failure to undergo isotype switching. Finally, we tested whether such immune changes affect survival in the face of challenge with a bacterial pathogen. Despite the defects in B cell numbers and antibody response, immunization affords CRH-tg mice equal protection against challenge with *S. pneumoniae* compared with wild-type (WT) mice. This may be due, in part, to an enhancement of innate immunity, as we found *augmented* numbers of neutrophils in CRH-tg mice.

Materials and Methods

Mice

The creation of CRH-tg mice was previously described (48). CRH-tg mice were subsequently back-crossed nine generations onto the C57BL/6 background. Mice were bred and housed in the specific-pathogen free facility at the Oregon Health Sciences University Department of Comparative Medicine. All procedures were approved by the Institutional Animal Care and Use

Committee (IACUC) of Oregon Health Sciences University. Experiments were performed with minimal psychological and physical stress to the animals. Mice were used at 2-4 months of age.

HPA response to restraint

Mice were housed overnight in pairs in shrouded cages to minimize environmental stress. Between 8 and 10 AM the following morning mice were restrained for 10 minutes in 50 ml conical tubes equipped with airholes. Blood was collected from the retro-orbital plexus at the indicated times in tubes containing EDTA (7.5 mg/ml). Cells were pelleted from collected blood and the remaining plasma was stored at –20°C until the assay date. CORT (ICN, Costa Mesa, CA) or ACTH (Nichols Institute Diagnostics, San Juan Capistrano, CA) was measured by commercial RIA or IRMA, respectively, according to the manufacturer's instructions.

Immunizations

For immunizations to examine both antibody titers and protection against *S. pneumoniae*, mice were injected i.p. with 70-100 ug phosphocholine coupled to keyhole limpet hemocyanin (PC-KLH) in 200ul CFA (primary immunization, day 0) or IFA (secondary immunization, day 14). Blood was collected for antibody measurement at days 0, 7, 19, and 26 and serum was frozen at -20°C until the assay date.

Streptococcus pneumoniae challenge

Naïve or PC-KLH immunized mice were infected with varying doses of *S. pneumoniae*, injected i.p. in 100 ul sterile saline. For PC-KLH immunized mice, bacterial challenge occurred seven days after the secondary immunization (day 21). Survival was analyzed statistically using Chi-square on collapsed doses, comparing total number of surviving mice: WT naïve versus CRH-tg naïve, WT immunized versus CRH-tg immunized, and naïve versus immunized within each genotype.

Streptococcus pneumoniae culture

S. pneumoniae (Wu-2, a generous gift from Dr. J. Kenney) were periodically passaged through WT mice to maintain a virulent stock. Bacteria were grown overnight on TSA/5% blood agar plates (PML microbiologicals, Wilsonville, OR). The following day, 5 ml of Todd-Hewitt's broth supplemented with 0.5% yeast extract (THY) and 0.2% sheep blood were inoculated with an individual colony. This culture was incubated 12 hours and then used to inoculate 125 ml of THY. Bacteria were collected at log phase, washed once, and dilutions made in sterile PBS. Precise enumeration was calculated retrospectively by plating bacterial dilutions on TSA/5% blood agar plates.

Leukocyte isolation

Bone marrow, spleen, and thymus were dissociated into single cell suspensions, washed twice in PBS and leukocytes counted via Trypan Blue exclusion to determine viable cell numbers. After

red blood cell lysis, peripheral blood leukocytes were washed three times in PBS and counted as above. These cells were then immunostained for FACS analysis as described below.

FACS analysis

Cells were pre-incubated with α-CD16/CD32 (Fc block, Pharmingen, San Diego, CA) to decrease Fc receptor-mediated background staining and subsequently incubated in PBS/3%FBS with the following panel of antibodies or appropriate isotype controls: α-CD3, α-CD4, α-CD8, α-CD45R (B220), α-CD43, α-CD23, α-IgM, α-IgD, α-CD11b (Mac-1), and α-Ly6G (1A8). B cell developmental stages were distinguished as follows: pro ($B220^{lo}$, $CD43^{+}$), pre ($B220^{lo}$, $CD43^{lo}$), immature (IgM^{+}, IgD^{-}), and mature (IgM^{+}, IgD^{+}) (161, 239-241). All antibodies except 1A8 were purchased from PharMingen (San Diego, CA) as direct conjugates to FITC, PE, CyChrome, or biotin. 1A8 (a generous gift from T. Malek) is a rat monoclonal antibody (IgG2a) that recognizes Ly6G (242), a differentiation marker restricted to neutrophils. The antibody bound to cells was detected with FITC-anti-rat IgG2a (PharMingen). Cells were fixed in 1% paraformaldehyde and analyzed the following day by three-color flow cytometry on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ).

Antibody measurement

Serum obtained from retro-orbital blood samples was stored at –20°C until the time of assay. Quantitative ELISA was performed to measure PC-specific or KLH-specific antibodies as previously described (243). Briefly, 96-well plates were coated overnight with PC-histone or KLH (1 ug/ml). Dilutions of individual or pooled sera were added and incubated for 1.5 hours at room

temperature, plates washed and samples detected by incubation with isotype-specific alkaline phosphatase conjugated secondary antibodies (Zymed, San Francisco, CA). p-Nitrophenyl phosphate substrate (Sigma, St. Louis, MO) allowed color detection at OD_{410}. Standard curves generated with T15 idiotype (PC-specific) monoclonal antibodies generated in our laboratory were used to determine the concentration of PC-specific antibodies in serum dilutions. For IgG determination a mixture of IgG1, IgG2a, IgG2b, and IgG3 T15 antibodies was used. For PC inhibition studies, all wells contained 0.2 M free PC or control diluent (0.1 M phosphate buffer).

Results

CRH-tg mice have decreased cellularity and altered leukocyte populations in primary and secondary lymphoid organs

We analyzed the cellularity and leukocyte populations in primary and secondary lymphoid organs of naïve CRH-tg and WT mice and found striking reductions in cellularity in the blood, spleen, thymus, and bone marrow of CRH-tg mice (Fig. 2). Spleen and thymus cell numbers were decreased 25 and 50-fold, respectively, consistent with effects previously reported with chronic HPA activation (244). Cellularity in the bone marrow and blood was also decreased, albeit to a lesser extent.

To test whether specific lymphocyte populations were preferentially decreased, we analyzed blood, spleen, and bone marrow by 3-color FACS analysis to detect relative proportions of B and T lymphocytes and B lymphocyte precursors. The percentage of total B cells was decreased by 2- to 3-fold in the spleen and blood of CRH-tg mice (Fig. 3*A* and *B*, left panels). Staining for IgM and IgD expression showed that this difference was primarily due to a loss of mature (IgD^+) B

cells, as seen when plotted as a percentage of total B cells (Fig. 3, *A* and *B*, right panels). We examined B cell precursors in the bone marrow to determine if this loss of peripheral B cells might result from altered hematopoeisis. Total B cells in the bone marrow were decreased more dramatically (~6-fold) than in the periphery (Fig. 4*B*, left panel). We further identified pro ($B220^{lo}$, $CD43^{+}$), pre ($B220^{lo}$, $CD43^{lo}$), immature (IgM^{+}, IgD^{-}), and mature (IgM^{+}, IgD^{+}) B cells using stage-specific markers (Fig. 4*B*, right panel) and found a near complete loss of pre B cells in CRH-tg mice (Fig. 4*A*). In fact, as a percentage of total B cells, only the pre B cell population was diminished (Fig. 4*B*). Thus, chronic HPA activation appears to affect mature B cells in the spleen and blood and developing B cells in the bone marrow as indicated by the loss in pre-B cells.

We also examined peripheral T cell populations since we were interested in T dependent antibody responses. Unlike B lymphocytes, the percentage of circulating T cells was similar between WT and CRH-tg mice (Fig. 3*B*, left panel). Moreover, in the spleen CRH-tg mice had a greater proportion of T cells (Fig. 3*A*, left panel), although the absolute number of T cells was still decreased. The CD4/CD8 ratio was not altered in these mice (data not shown). Thus, alterations in T cells populations are unlikely to contribute to an antibody defect, although we have not ruled out the possibility of altered T cell function.

CRH-tg mice exhibit an altered antibody response to PC-KLH immunization

To investigate the reported defect in antibody production in CRH-tg mice, we examined primary and secondary antibody responses to a thymus-dependent antigen, PC-KLH. PC is an important antigenic component of cell-wall polysaccharides of numerous pathogens including Gram positive bacteria (*S. pneumoniae*), Gram negative bacteria (*H. influenzae*, Salmonella), protozoans

(Leishmania, Trypanosomes), and parasites (tapeworm, nematodes) (245). Antibodies against PC have been shown to be protective against pneumococcal and filarial infection (246-251). Thus, we elected to immunize mice with PC coupled to a protein carrier, keyhole limpet hemocyanin (KLH) which allowed us to examine thymus-dependent antigen responses and test whether immunization results in improved protection against a relevant pathogen, *S. pneumoniae.*

We found that pre-immune titers of anti-PC IgM antibodies were actually slightly higher (~2-fold) in CRH-tg mice than in WT mice (Fig. 5*A*). In addition, CRH-tg mice had primary (day 7) and secondary (days 19, 26) anti-PC IgM responses that were robust, albeit somewhat lower (~3-fold) than WT mice. Primary (day 7) anti-PC IgG titers were low in both genotypes, but following secondary antigen challenge (days 19, 26) WT mice mounted a strong IgG response. This response was severely decreased (10-fold below WT levels) in CRH-tg mice, indicating impaired isotype switching (Fig. 5*B*). This impairment is not due simply to delayed kinetics as IgG titers after immunization did not rise above levels seen on day 26 (followed out to 4 months post-secondary challenge; data not shown). We observed similar decreases in IgM and IgG anti-KLH antibody titers, demonstrating that this alteration is not unique to the PC hapten response (data not shown).

The antibodies produced in response to PC-KLH also differed in fine specificity in the CRH-tg mice. WT mice exhibited normal characteristics of the antibody response to PC-KLH, namely that the response was initially dominated by antibodies that bind both PC-protein (or nitrophenyl PC, NPPC) and free PC. The majority of these antibodies are known to be T15 idiotype positive (VH1/Vκ22) and protective against *S. pneumoniae* (248, 249). Following secondary challenge new clones emerge that do not bear the T15 idiotype and fail to bind free PC, but retain specificity for NPPC (243). These latter antibodies do not protect against *S. pneumoniae* because they do not

bind the bacteria. Such NPPC-restricted antibodies eventually represent ~50% of the anti-PC-protein response in C57BL/6 mice (252). This antibody profile is shown in Fig. 5*C*, in which 90% of WT antibodies obtained early in the response are inhibitable by free PC. However, by day 19 only 50% of the antibodies are inhibitable by free PC, indicating a shift in antibody specificity over time. In contrast, CRH-tg mice fail to show this shift in fine specificity; >90% of the antibodies in both the primary and secondary response are inhibitable by free PC. To ensure that the antibodies from both genotypes were specific for PC in the context of protein, we tested them for binding to NPPC, a hapten analog which mimics the linkage structure of PC to the protein carrier. Antibodies from both genotypes were ~100% inhibitable by NPPC, confirming the specificity for PC-protein (data not shown). Thus, the pattern of antibody fine specificity indicates that as the response to PC-protein matures CRH-tg mice retain antibodies that are inhibitable by free PC and do not generate antibodies that are restricted to PC-protein. This suggests that these animals may differ from WT mice in clonal selection and V gene usage.

CRH-tg and WT mice are equally susceptible to infection with S. pneumoniae

The decrease in B lymphocytes and poor antibody responses after immunization suggested that CRH-tg mice would be more susceptible to infection with *S. pneumoniae*, a Gram positive pathogen that requires opsonization for efficient clearance (253). We challenged CRH-tg and WT mice with various doses of a virulent strain of *S. pneumoniae*, Wu-2, to determine whether CRH-tg mice had increased susceptibility. Unexpectedly, survival was not different between naïve mice of both genotypes ($p<0.3$, Chi-square, Table 1). Furthermore, most animals died between 2 and 4 days after infection irrespective of genotype, indicating that the kinetics of death were similar between the two groups (data not shown). We then tested whether immunization with PC-KLH,

which is known to induce protective antibodies in WT mice (254, 255), would provide resistance against *S. pneumoniae* challenge in CRH-tg mice. Surprisingly, immunization protected CRH-tg and WT mice equally well (>100-fold compared with naïve) (Fig. 6, Table 1). Again, there were no differences in survival between immunized CRH-tg and WT mice.

Augmented neutrophils in CRH-tg mice

It was unexpected to find that immunization afforded CRH-tg and WT mice equal protection against *S. pneumoniae* given the diminished B cell numbers and antibody responses. Antibodies are critical in clearing *S. pneumoniae* because opsonization allows phagocytic cells, particularly neutrophils, to engulf bacteria by binding via Fc and complement receptors (253, 256-260). Thus, we speculated that the protection in CRH-tg mice might result from increases in neutrophils that could compensate for decreased antibody titers. We examined myeloid and granulocyte populations using the specific markers CD11b and Ly6G. Both monocyte/macrophages and neutrophils express CD11b, whereas Ly6G is neutrophil-specific (242). Monocyte/macrophage populations were similar in CRH-tg and WT bone marrow and blood, and these cells were increased in CRH-tg spleen (Fig. 7*A*). This is likely due to the B cell depletion, wherein a decrease in one population results in a proportional increase in others. The percentage of neutrophils was increased in CRH-tg mice in all lymphoid tissues examined (Fig. 7, *B* and *C*). Moreover, the increase in circulating neutrophils was so great (>10-fold) that despite the overall decreased cellularity, CRH-tg mice had greater *numbers* of circulating neutrophils per volume of blood compared to WT mice (Fig. 7*D*). Thus, despite decreased lymphocytes, neutrophil populations were increased in CRH-tg mice.

Until recently, the immunological consequences of stress have been viewed as largely detrimental. It is becoming increasingly evident that this view does not convey the complete picture and is inaccurate in certain situations. Decreased lymphocyte numbers occur following acute and extended periods of stress, but such diminution does not always lead to suppressed functional outcomes of immune defense. Furthermore, new evidence supports the idea that exposure to acute stress can lead to enhanced immune responses (120, 121). Some have proposed that while acute stress can be immunoenhancing, chronic stress is detrimental (261). Our findings with susceptibility to *S. pneumoniae* suggest that this may not always be true. Thus, while stress-induced modulation of the immune response alters certain components of the immune system (e.g. cell numbers, population composition), the consequences on immune function need to be analyzed individually. Furthermore, effects of stress on innate and adaptive aspects of the immune system are not necessarily synchronous; thus, suppression of adaptive responses may be counterbalanced by enhancement of certain innate components.

Using a genetic model of chronic HPA activation, we found strong evidence for changes in both adaptive and innate components of the immune system. Stress-induced decreases in lymphoid numbers have been a fairly consistent finding among most models of stress and HPA activation. We found that CRH-tg mice exhibit decreased lymphoid numbers in all immune compartments examined. This may be due to increased glucocorticoids which have been shown to decrease the size of the spleen and thymus, although decreased cellularity has not been observed consistently in the blood and bone marrow (113, 262-265). Furthermore, lymphocytes are known to be particularly sensitive to glucocorticoid-mediated apoptosis; thus, we speculate that the decrease in

T and B lymphocytes in CRH-tg mice is due to increased basal levels of corticosterone (109, 112, 113, 158). It is noteworthy that CRH-tg mice show a profound reduction in pre B cells in the bone marrow, which to our knowledge is the first evidence that chronic HPA activation *preferentially* depletes pre B cells. Again, this is likely due to excess glucocorticoids as delRey and colleagues (108) showed that dexamethasone treatment induced widespread loss of pre B cells, but had a lesser effect on pro and mature B cells. Interestingly, double positive (DP) T cells, which are at a developmental stage analogous to pre B cells, are well known for their susceptibility to glucocorticoid-mediated apoptosis (158).

Precursor lymphocytes may be affected preferentially by glucocorticoids because these hormones play a role in lymphocyte selection. In the case of $CD4^+/CD8^+$ T cells, glucocorticoids antagonize TCR-mediated apoptosis which allows selection of clones with low to moderate avidity for self antigens. Without glucocorticoid signaling at this stage, the threshold for positive selection is raised and T cells with low to moderate avidity for self antigens die (266, 267), although the total number of DP T cells generated in the thymus remains unchanged (268). In the case of pre B cells, it is not known whether glucocorticoids influence B cell selection. However, our results showing that pre B cells are particularly sensitive to chronic HPA activation raise this intriguing possibility. Furthermore, there is a marked difference in the antigen specificity of B cells induced by PC-KLH when comparing CRH-tg and WT mice. This difference in fine specificity quite likely indicates a difference in responding B cell clones and thereby suggests that the B cell repertoire may be altered during chronic HPA activation. Additionally, this observation is consistent with a failure to develop a strong memory response since NPPC-restricted antibodies normally arise during secondary and memory responses to PC-KLH.

Depletion of mature B cells in the spleen was also evident among CRH-tg mice. This was unanticipated given that mature B and T cells are relatively resistant to glucocorticoid-mediated apoptosis (160, 264, 265). This could be a reflection of the decrease in B cell precursors, leading to fewer cells capable of entering the mature B cell pool. If this were the case, however, we would expect the proportion of immature B cells in the periphery to be similarly depleted. It is conceivable that hormones that act upstream of glucocorticoids such as CRH and ACTH may affect mature B cells in a manner that is independent of glucocorticoids (85, 232, 269, 270). In addition to direct effects on mature B cells, it is possible that chronic HPA activation leads to changes in specific factors necessary for B cell survival thus indirectly limiting their development and/or expansion.

Adaptive responses are also impaired as measured by the ability of these mice to generate antibody responses to the antigen, PC-KLH. CRH-tg mice mount an IgM response to PC-KLH immunization but exhibit poor isotype switching. The precise mechanism underlying this impairment is not clear at this time. It is reasonable to speculate that B cells in these animals are unable to respond to appropriate signals needed to induce isotype switching or memory cell formation, perhaps due to altered expression of cytokine receptors or inhibition of signaling pathways (271). Lack of adequate T cell help, a critical component for isotype switching, may also be a factor. This could result from a decrease in T cell number, making cell to cell interactions infrequent. We think this is unlikely, since the T cell:B cell ratio is actually higher in the spleens of CRH-tg mice (data not shown). Instead, the T cells making up this population may be actively regulating B cell function. Alternatively, CRH-tg T cells may not express adequate levels of costimulatory molecules, such as CD40L, or produce cytokines appropriate for class switching (272).

Surprisingly, immunization with PC-KLH provided CRH-tg and WT mice equal protection against *S. pneumoniae*. Given the low B cell numbers and poor antibody response we predicted that immunization would be less beneficial to CRH-tg mice. However, since this was not the case, we need to consider that even the low level of antibodies achieved in CRH-tg mice is sufficient to opsonize and clear *S. pneumoniae*. Immunization of WT mice with PC-KLH may elicit antibody titers that exceed those needed to protect against *S. pneunomiae*. However, previous studies have shown that when mice are immunized with pneumococcal polysaccharide, individual antibody titers correlated with survival in mice challenged with *S. pneunomiae*, indicating in that system antibody levels are *not* in great excess (273). It is also possible that IgM antibodies play a more important role in bacterial clearance than has been appreciated previously. If this were the case, CRH-tg mice would be reasonably well protected given that their levels of anti-PC IgM were only modestly reduced. Indeed, an early study showed that *S. pneumoniae* pre-opsonized with IgM antibodies obtained from pneumococcus-immunized animals were cleared more rapidly from the bloodstream than those pre-opsonized with IgG (253). More recently, a number of studies have shown a critical role for IgM antibodies in other models of bacterial infection (274-276). These observations, taken together with the recent isolation and identification of a novel Fc receptor for IgM that enhances opsonic uptake (277), support a vital role for IgM in defense against bacterial disease, particularly in the early phases of infection that involve phagocytosis and clearance of bacteria.

It is also reasonable to speculate that augmented innate defenses may compensate for the lower antibody titers, allowing clearance of bacteria that are not opsonized optimally. We found that CRH-tg mice have increased numbers of circulating neutrophils, which may afford significant

protection in the face of diminished antibody responses. The increase in neutrophils seen in these animals is likely due to elevated corticosterone levels since glucocorticoids have been shown to increase the production, release from the bone marrow, and half-life of neutrophils (115, 116, 278, 279). Future studies are necessary to determine if specific functions of innate immunity are enhanced in CRH-tg animals.

The idea that innate immune mechanisms may be augmented in CRH-tg mice is supported by other stress-related studies. Both stress and glucocorticoids have been shown to increase the phagocytic activity of neutrophils and macrophages (280-288). In addition, glucocorticoids induce molecules vital to opsonization such as complement and C-reactive protein, an acute phase protein that binds PC and is protective against *S. pneumoniae* (289-294). Other products of the stress response, such as CRH, may also modulate innate immunity. Recent studies have shown that CRH receptors are expressed on splenic macrophages and can be induced on neutrophils following immunization or restraint stress (81, 82, 295). In the case of macrophage function, CRH has been shown to increase superoxide production (296), suggesting that an innate mechanism of cytotoxicity could be enhanced by increased CRH production during stress.

Collectively our studies suggest that during stress, innate immune mechanisms are preserved while adaptive responses are clearly compromised. A balance appears to be achieved by these changes because CRH-tg mice develop an adequate immune response to protect against challenge with a lethal pathogen despite profound loss of B lymphocytes and diminished antibody responses. During periods of stress there are likely to be important demands on the immune system due to infection, thus stress regulation of the immune response may have evolved to provide a well-orchestrated immune defense to ensure survival.

Acknowledgements

We thank Janet Duncan for technical assistance and McKay Brown for expert technical advice. We are also grateful to Greg Wiens and Sarah Coste for critical review of the manuscript and Susan Stevens for insightful discussion.

Table 1. Naïve CRH-tg and WT mice have similar survival rates after infection with *S. pneumoniae*

	# of bacteria		
	1	10	100
WT	5/9[a]	1/9	3/9
CRH-tg[b]	3/8	3/9	0/9

[a] number of mice that survived/ number of mice infected

[b] CRH-tg not statistically different from WT, $p>0.3$, Chi-square

Footnotes

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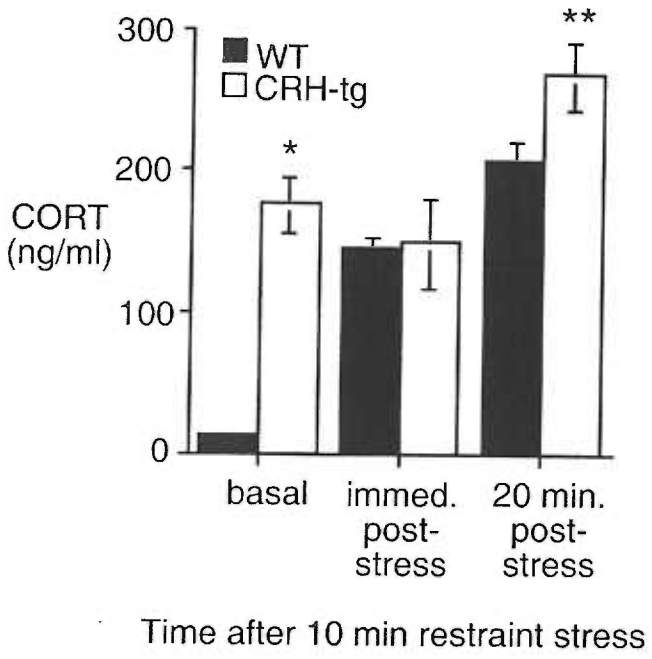

Figure 2-1. CRH-tg mice have increased basal levels of CORT, which increase further in response to restraint stress. Mice were restrained for 10 minutes in 50 ml polypropylene conical tubes. Blood was collected from the retro-orbital plexus at the following times: basal (unstressed), immediately after restraint, or 20 minutes following cessation of restraint. Results are the mean ± SEM of 8 mice per genotype; each mouse was bled at one time point only. (*$p<0.05$, CRH-tg vs. WT; **$p<0.05$, CRH-tg 30 min. vs. CRH-tg basal; Newman-Keuls post hoc).

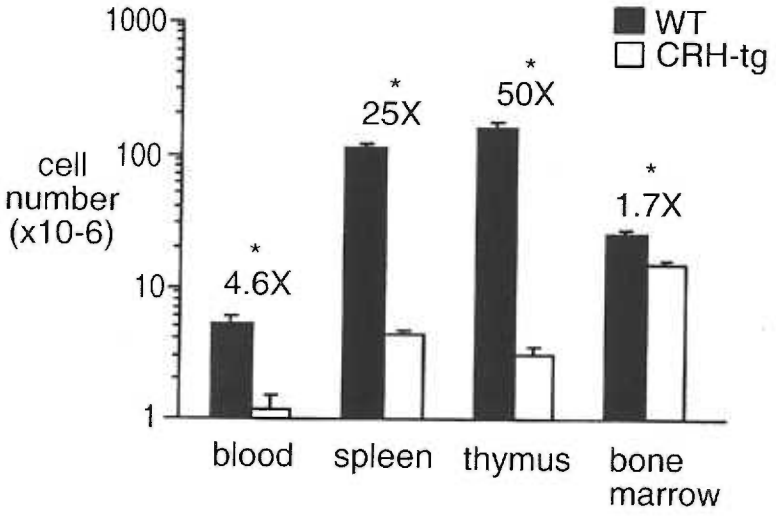

Figure 2-2. CRH-tg mice have decreased cellularity in lymphoid tissues. Spleen, thymus, and bone marrow were dissociated into single cell suspensions and leukocytes counted by Trypan Blue exclusion. Results are the mean ± SEM of 8 mice per genotype (*$p<0.05$; Student's t test).

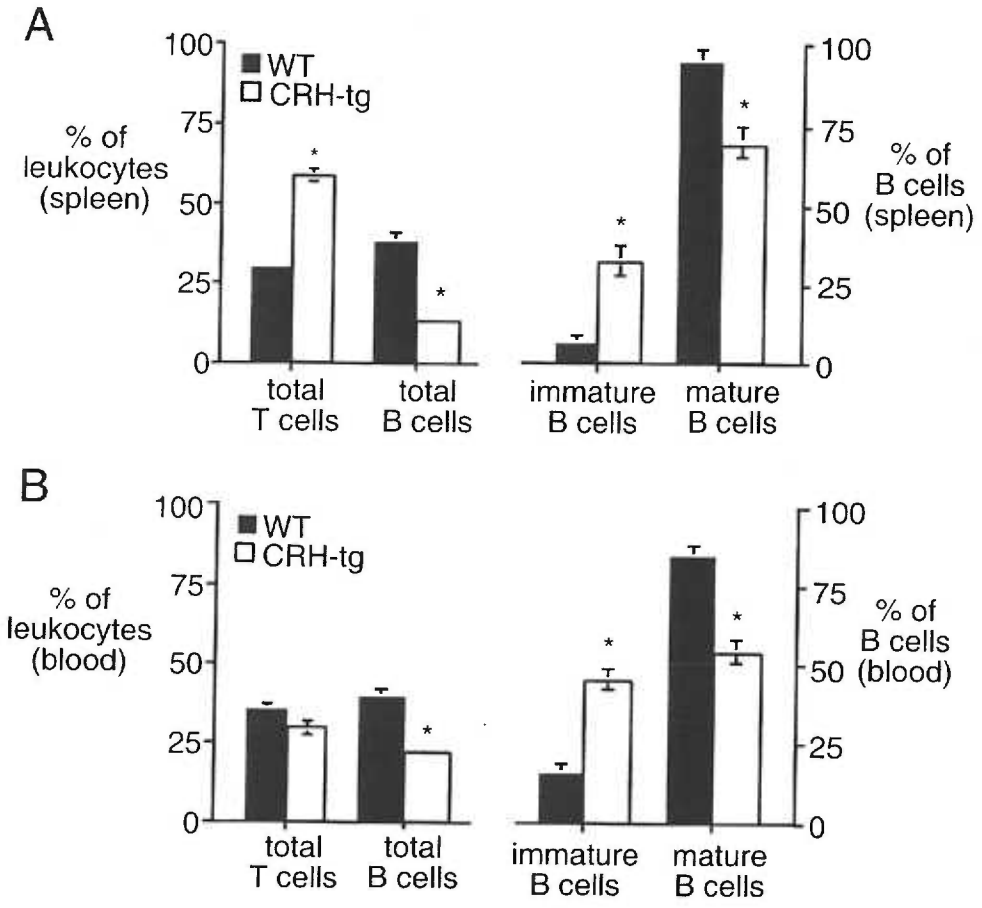

Figure 2-3. CRH-tg mice have altered T and B cell populations in peripheral lymphoid tissues. Splenocytes and peripheral blood leukocytes (*A* and *B*, respectively) were processed as described in Figure 2. Leukocytes (105-106 per tube) were then stained with monoclonal antibodies that recognize CD3, B220, IgM, and IgD and analyzed by 3-color FACS. Results are the mean ± SEM of 8 mice per genotype (*$p<0.05$; Student's *t* test).

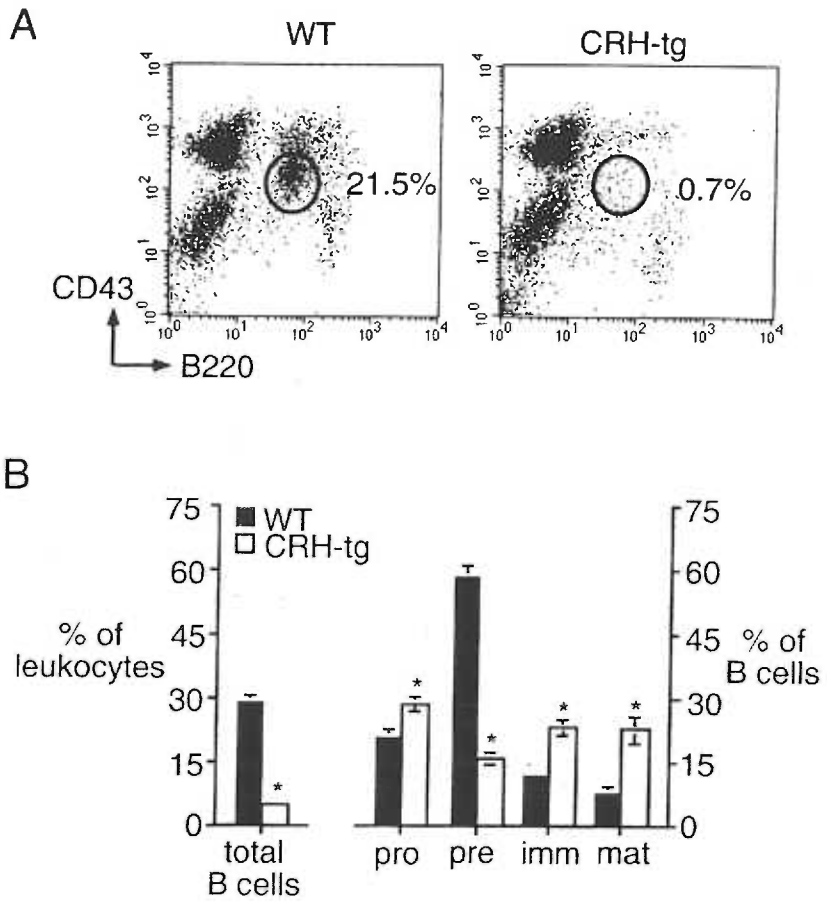

Figure 2-4. CRH-tg mice display a selective loss of pre B cells in the bone marrow. Single-cell suspensions of bone marrow cells were stained with a panel of antibodies to distinguish B cell developmental stages: pro (B220lo, CD43+), pre (B220lo, CD43lo), immature (IgM+, IgD-), and mature (IgM+, IgD+). CRH-tg mice have a selective loss of pre B cells as seen by low B220 and low CD43 staining, *A*, and when analyzed as a percentage of total B cells, *B*. Results are the mean ± SEM of 8 mice per genotype (*$p<0.05$; Student's *t* test).

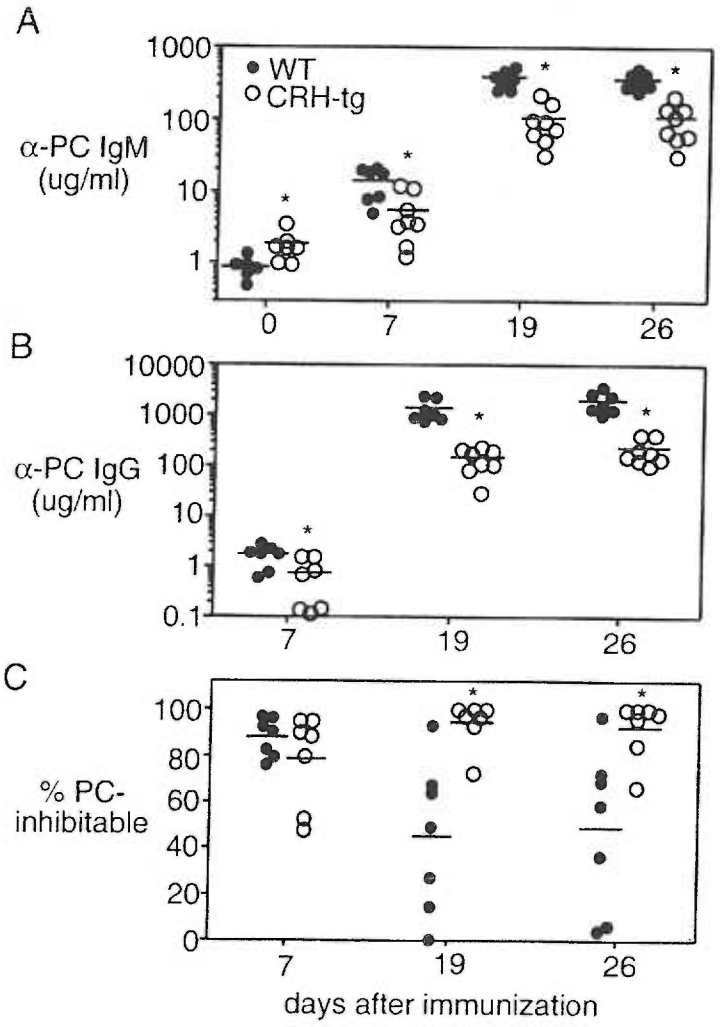

Figure 2-5. Following PC-KLH immunization, antibody responses in CRH-tg mice are decreased in titer and differ in specificity compared with WT mice. Mice were immunized with PC-KLH (70 ug in CFA) and boosted on day14 (70 ug in IFA). Serum was collected at the indicated time points and analyzed for binding to PC-protein by quantitative ELISA. ELISA plates were coated with PC-histone and antibody binding was detected with anti-IgM-AP, *A*, or anti-IgG-AP, *B* and *C*. For PC inhibition, wells contained 0.2M free PC or control buffer, *C*. (*$p<0.05$; Student's *t* test).

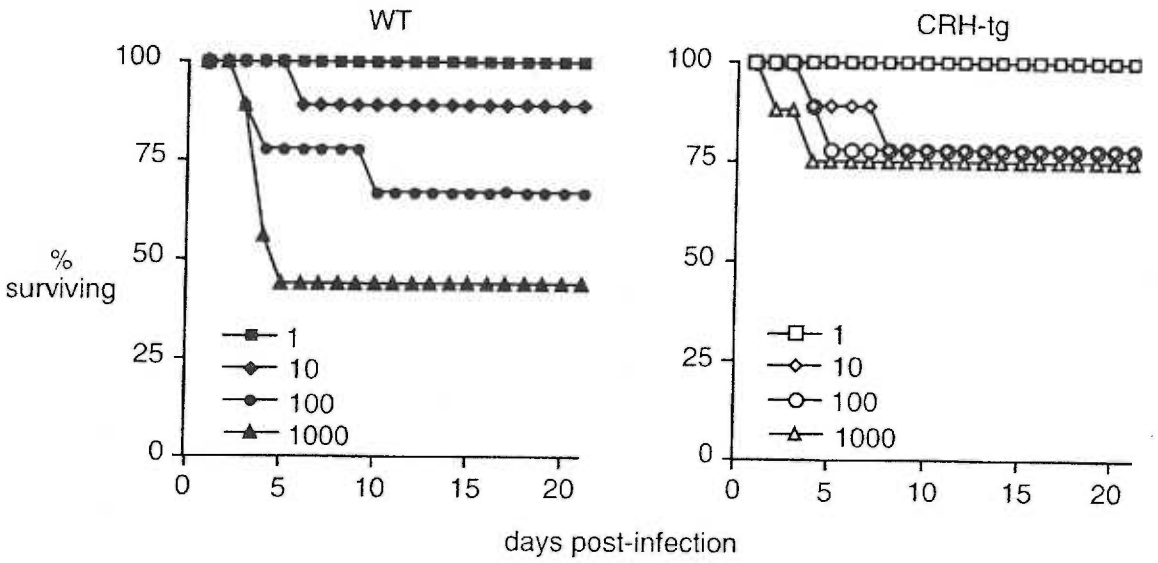

Figure 2-6. CRH-tg mice survive *S. pneumoniae* infection equally well compared with WT mice. PC-KLH immunized mice (9-10 mice per dose per genotype) were challenged i.p. with doses of 1, 10, 100, or 1000 *S. pneumoniae* organisms per mouse. There was no significant genotype difference in percent of mice surviving or average day of death. Both genotypes were significantly protected (~100-fold) by immunization ($p<0.05$; Chi-square).

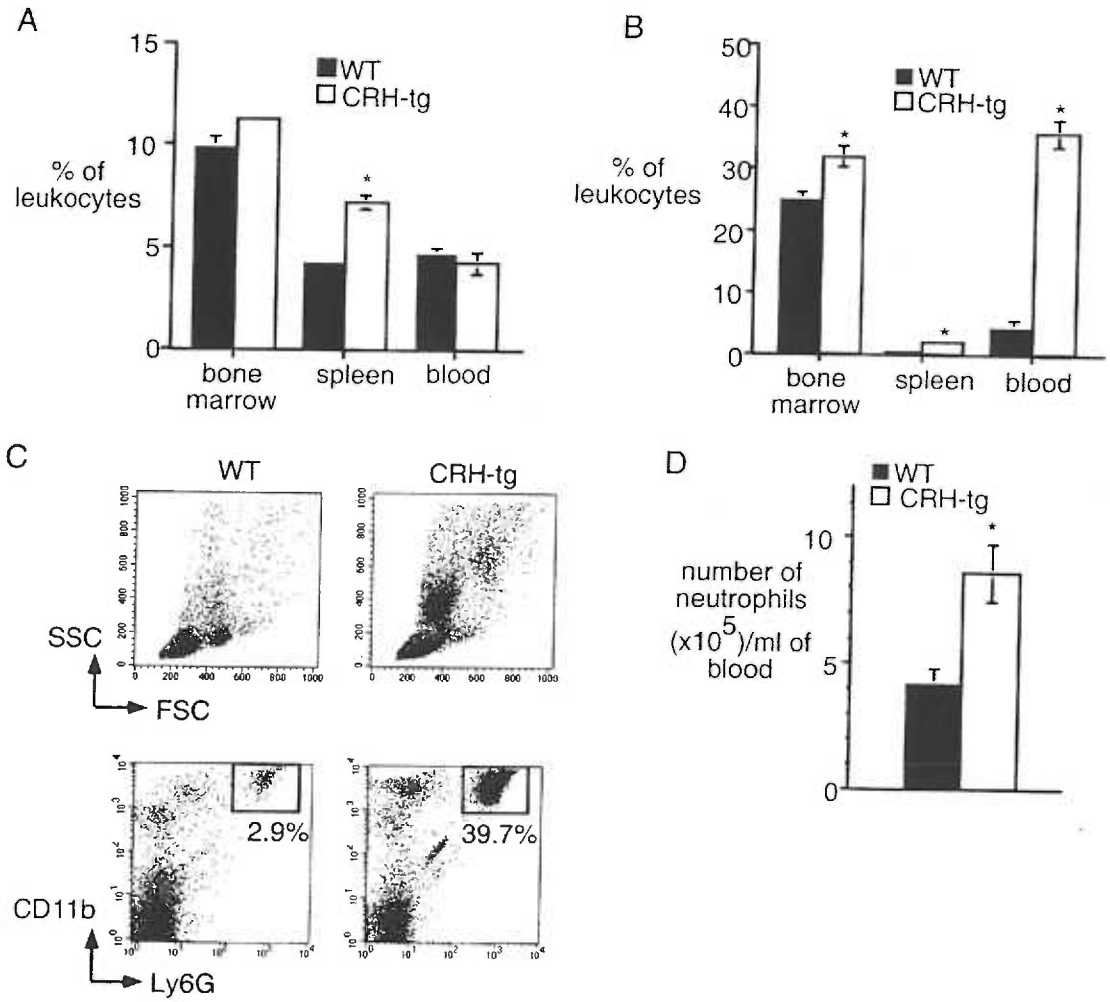

Figure 2-7. CRH-tg mice have increased numbers of granulocytes. Single-cell suspensions of spleen, blood, and bone marrow were stained with monoclonal antibodies to CD11b and Ly6G to distinguish monocyte/macrophages versus neutrophils, *C* (lower panels). Neutrophils were also distinguished by high side scatter, *C* (upper panels). CRH-tg mice had an increased proportion of monocyte/macrophages in the spleen, *A*, and increased proportion of neutrophils in all tissues examined, *B* and *C*. The absolute number of blood neutrophils was obtained by multiplying the percentage of neutrophils by the number of leukocytes per ml of blood, *D*. Results are the mean ± SEM of 8 mice per genotype (*$p<0.05$; Student's *t* test).

Chapter 3—Manuscript #2

Glucocorticoids block germinal center formation and diminish follicular dendritic cell networks in the spleen.

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Abstract

During the response to stress, hypothalamic-pituitary activation causes the adrenal gland to release glucocorticoids. These hormones alter energy metabolism and are key regulators in the neuroendocrine and immune systems. We described previously a mouse model of chronic hypothalamic-pituitary-adrenal activation in which overproduction of a corticotropin-releasing hormone (CRH) transgene in the central nervous system leads to increased circulating corticosterone. CRH transgenic mice mount relatively normal primary immune responses to thymus-dependent antigen, but following antigen boost, isotype switching is impaired and antibody specificity is skewed. These striking humoral abnormalities led us to investigate germinal center formation in CRH transgenic mice. We find that after immunization, wild-type mice form abundant germinal centers in the spleen that persist past three weeks, while CRH transgenic mice fail to form germinal centers. Moreover, inhibition of germinal center formation is recapitulated in wild-type mice by chronic corticosterone administration. Regulation of germinal centers by corticosterone appears to be mediated, in part, through effects on follicular dendritic cells, because staining of follicular dendritic cell networks is decreased as is the capacity to trap antigen-antibody complexes in follicles. These data suggest a novel mechanism whereby glucocorticoids may significantly impair humoral immune responses.

Key words: antigen-antibody complex, immunosuppression, antibody formation, corticosterone, stress

Introduction

The hormonal response to stress is initiated through hypothalamic-pituitary activation, which culminates in the release of adrenal glucocorticoids (cortisol in humans, corticosterone in rodents). Glucocorticoids are key regulators in the stress response via mobilization of energy stores and negative feedback of the primary hypothalamic-pituitary stress circuit (reviewed in (3)). Synthetic glucocorticoids (prednisolone, dexamethasone) have long been appreciated as potent anti-inflammatory agents, and in clinical settings their therapeutic use spans a broad range of pathologic conditions such as rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, autoimmune disorders, and transplant rejection. However, long-term administration of glucocorticoids or chronic elevations in cortisol can leave individuals immunosuppressed, and thus at risk for infectious disease and slow to heal injuries (140, 231).

Effects of glucocorticoids on the immune system include leukocyte apoptosis and altered cytokine responses due to a shift from Th1 to Th2 (297, 298). Developing B and T lymphocytes are particularly sensitive to glucocorticoid-induced apoptosis (109, 111, 113). However, the effects of these steroid hormones on survival and activation of mature B cells are not well studied, particularly with regards to formation and maintenance of antibody responses.

We developed a transgenic mouse model of chronic hypothalamic-pituitary-adrenal (HPA) activation in which corticotropin-releasing hormone (CRH) is overexpressed in the CNS (299). These mice (CRH-tg) have high circulating levels of corticosterone due to chronic HPA activation and display physiologic features of Cushing's syndrome, typical of patients treated with chronic systemic glucocorticoids. We reported previously that these mice have severe perturbations in

leukocyte populations, with predominant decreases in B cell populations (1). In addition, while their pre-immune antibody titers and primary IgM responses to immunization are relatively normal, they display poor isotype switching and an altered antibody repertoire upon secondary immunization. These latter changes indicate that germinal centers could be altered in CRH-tg mice because germinal centers provide an environment that supports B cell proliferation, isotype switching and affinity maturation of antigen-specific clones during an immune response (300, 301). Thus, we postulated that increased corticosterone alters germinal center formation, perhaps through effects on follicular dendritic cells (FDC), which trap antigen-antibody complexes during an antigen driven response.

Here we report that CRH-tg mice with chronic elevations in circulating corticosterone show poor germinal center formation after immunization. We also demonstrate that impaired germinal center formation occurs in mice treated with exogenous corticosterone and that compromised FDC networks may underlie this effect.

Materials and Methods

Mice

The generation of CRH-tg mice was described previously (299). CRH-tg mice were back-crossed nine generations onto the C57BL/6 strain. C57BL/6 mice were purchased from NCI. All mice were housed under specific pathogen free conditions and were given free access to laboratory chow and water. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University. Mice were used at 2-4 months of age.

Corticosterone treatment

Corticosterone (Sigma, St. Louis, MO) was dissolved in 0.8% ethanol and administered in the drinking water. Control mice received vehicle only (0.8% ethanol) in their drinking water. Mice were bled weekly to monitor serum corticosterone levels.

Corticosterone measurement

Blood for plasma corticosterone measurement was obtained from the retro-orbital plexus and collected in 40 uL EDTA (7.5 mg/ml). Mice were bled between 08:00 and 10:00 within 90 seconds of disturbing the cage in order to minimize any unintended environmental stress. Cells were pelleted and the remaining plasma was stored at –20°C until assay by commercial RIA (ICN, Costa Mesa, CA) according to the manufacturer's instructions.

Immunization and tissue collection

Mice were injected i.p. with phosphocholine coupled to keyhole limpet hemocyanin (PC-KLH, 70 ug total protein) or with 200 uL defibrinated sheep reed blood cells (SRBC) (Colorado Serum Company, Denver, CO) diluted 1:10 in HBSS. PC-KLH was synthesized in our laboratory as described (302) and emulsified in a total of 200 uL Complete Freund's Adjuvant (CFA, Invitrogen, Carlsbad, CA). For antibody titers (Table 1) mice received an i.p. antigen boost of 70 ug PC-KLH in Incomplete Freund's Adjuvant (IFA, Invitrogen) 14 days after the primary immunization. Blood was collected at the indicated time points, and serum was frozen at –20°C until assay by ELISA. Spleens were collected 7, 13, or 22 days after primary immunization for

germinal center and in vitro peroxidase-anti-peroxidase immune complex (PAP) analysis. For in vivo PAP experiments, naive mice were injected i.v. with 30 uL undiluted PAP (250 ug, Dako, Carpinteria, CA), and spleens were harvested 24 hours later. For all experiments, spleens were placed in cryomolds in cold OCT and frozen in isopentane chilled in liquid nitrogen. The blocks were then stored at –80°C until sectioning.

Antibodies

The following reagents were used for immunohistochemistry/immunofluorescence: peanut agglucitnin (PNA) (Vector, Burlingame, CA), goat-anti-PNA (Sigma), alkaline-phosphatase (AP) conjugated rabbit-anti-goat (Vector), FDC-M1 (BD PharMingen, San Diego, CA), anti-rat κ/λ-AP (Sigma), anti-rat γ2c-biotin (Caltag, Burlingame, CA), anti-mouse CD16/CD32 (FcγRII/III, BD PharMingen), anti-mouse CD21/CD35 (complement receptor, BD PharMingen), anti-rat γ2b-AP (BD PharMingen), FITC anti-IgM (Southern Biotechnology Associates, Birmingham, AL); rhodamine isothiocyanate anti-CD5 (BD PharMingen), MOMA-1 (Serotec, Oxfordshire, U.K.), goat-anti-rat IgG biotin, streptavidin-AMCA (Vector), PAP (Dako). AB1-2 hybridoma cells producing anti-T15 idiotype (VH1/Vκ22) mAb (303) were obtained from American Type Culture Collection (Rockville, MD), grown as ascites, and the antibody purified on a protein A-Sepharose 4B column.

Immunohistochemistry

Frozen sections of spleens were cut (10 um) onto poly-L-lysine treated slides and fixed in either cold acetone or 4% paraformaldehyde; slides were then stained immediately or stored at –80°C.

For colorimetric detection, tissue sections were blocked for 30 min in TNB blocking buffer (0.1 M Tris-HCl, 0.15 M NaCl, 5 mg/ml blocking reagent (tyramide amplification kit, Perkin Elmer Life Sciences, Boston, MA)). Sections were then incubated for one hour with primary antibody or PNA. Sections were washed 3x in wash buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.1% Tween-20), then incubated with horse radish peroxidase (HRP) or AP-conjugated secondary antibody. For PNA staining to detect germinal center B cells, the goat-anti-PNA secondary was detected with an AP-conjugated rabbit-anti-goat tertiary antibody. HRP and AP enzymatic activities were visualized with 3-amino-ethyl-carbazole (Sigma) and naphthol AS-MX phosphate (Sigma), respectively.

Immunofluorescence staining and analysis of naive spleens was performed as described (304).

Germinal center and PAP quantitation

Slides were scanned into digital format using a SlideScan 35 (Polaroid, Cambridge, MA). Number and size of germinal centers were calculated using NIH Image software (version 1.61).

Antibody measurement

Serum was stored at –20°C until the time of assay. Quantitative ELISA was performed to measure SRBC-specific and PC-specific antibodies. For PC-specific antibodies, 96-well plates were coated overnight with PC-histone (1 ug/ml) as described previously (1). Dilutions of individual or pooled sera were added and incubated for 1.5 hours at room temperature, plates washed and samples detected by incubation with rat α-mouse IgG-specific AP-conjugated secondary antibodies

(Zymed, San Francisco, CA). P-Nitrophenyl phosphate substrate (Sigma, St. Louis, MO) allowed color detection at OD_{410}. To determine the concentration of PC-specific antibodies in serum dilutions, standard curves were generated with T15 idiotype positive monoclonal antibodies (a kind gift from M.B. Rittenberg). For PC-inhibition, all wells contained 0.2 M free PC or control diluent (0.1 M phosphate buffer).

Relative SRBC-specific antibody titers were determined by the method of Mori and Koda (305). Briefly, 96-well plates were coated for one hour with 10^7 SRBC/well in PBS. Plates were centrifuged for 3 min at 2000 rpm, then inverted for ~30 min to remove non-adherent SRBC. Wells were washed 3x in PBS, then blocked with PBS/5% FBS/1% BSA. Serum was diluted 1:250 to 1:2000 for IgM determination and 1:5000 to 1:40,000 for IgG determination, added to replicate wells, and incubated 1 hour at room temperature. Wells were washed and samples detected by incubation with rat α-mouse IgM-specific or rat α-mouse IgG-specific AP-conjugated secondary antibodies. AP activity was visualized with disodium phenylphosphate substrate as described (305), allowing color detection at OD_{490}. The fold differences in titer between paired samples from vehicle and corticosterone-treated animals were assessed and sample means were compared by Student's *t* test. Data are expressed as fold difference between vehicle and corticosterone-treated titers.

Results

CRH-tg mice fail to form germinal centers upon immunization

We reported previously that CRH-tg mice immunized with PC-KLH develop primary and secondary anti-PC IgM responses that are fairly robust, whereas secondary IgG responses

following antigenic challenge are severely decreased (10-fold below WT levels), which indicates impaired isotype switching (1). Here, we extend the time course of this observation to 50 days post-immunization, at which point IgG titers in WT mice have peaked and are beginning to decline. At these late time points, IgG titers in CRH-tg mice remain >10-fold below those of WT mice (Table 1). This indicates that isotype switching is not simply delayed in CRH-tg mice, but rather remains depressed throughout the response. We saw a similar diminution of the anti-KLH secondary IgG response (data not shown), which demonstrates that this alteration is not unique to the PC hapten response. We also observed that the antibodies produced in response to PC-KLH differ in fine specificity between WT and CRH-tg mice (Table 1). In WT mice the response to PC-KLH is dominated initially by antibodies that bind both nitrophenyl-PC and free PC and which are primarily T15 idiotype positive (VH1/Vκ22). Following secondary challenge new clones emerge that fail to bind free PC, but bind PC-protein or nitrophenyl-PC with higher affinity than does T15 (243). Such antibodies, detected by the inability of free PC to inhibit binding to PC-protein, eventually represent ~50% of the anti-PC-protein response in C57BL/6 mice (252). CRH-tg mice fail to show this shift; >90% of the antibodies in both the primary and secondary response are inhibitable by free PC (Table 1). These data suggest that B cell responses in CRH-tg mice and WT mice may differ in clonal selection and V gene usage.

In light of the impaired isotype switching and altered clonal selection seen in CRH-tg mice, we hypothesized that germinal center formation may be abnormal. To examine this, we evaluated germinal center formation in the spleen following primary immunization with PC-KLH. Unimmunized mice of either genotype showed essentially no germinal centers (data not shown). However, spleens from immunized WT mice contained follicles with large, PNA^+ germinal centers whose numbers peaked at day 7 (Fig. 1, top left panel) (average of 63 per section) and

declined to ~26 and ~18 per section by days 13 and 22, respectively (data not shown). In contrast, we found a near absence of germinal centers in CRH-tg spleens at all time points tested (see Fig. 1 top right, day 7). Examination of nine CRH-tg spleens (among three time points) revealed the presence of only two small germinal centers in each of two CRH-tg mice, both at day seven.

We also tested whether the formation of T15 idiotype positive foci was altered in CRH-tg mice. We stained the same spleen sections with a T15 idiotype-specific antibody (AB1-2) to examine foci of PC-specific antibody producing cells. WT spleens showed intense foci of AB1-2 staining in follicles and red pulp (Fig. 1, top left panel). In contrast, CRH-tg mice showed 4-fold fewer AB1-2$^+$ foci (6.5$\pm$3.2/spleen section in CRH-tg mice vs. 28.2$\pm$2.9/spleen section in WT mice) on days 7 (Fig. 1, top right panel) and 13. Those foci found to be T15 idiotype positive were substantially smaller in CRH-tg mice than in WT mice (Fig. 1, top panels). Neither genotype exhibited staining with PNA or AB1-2 in unimmunized (naive) spleen sections (data not shown). Thus, while germinal centers are essentially absent in CRH-tg mice, foci of antigen-specific B cells are diminished, but not absent. This is consistent with our finding of decreased specific immunoglobulin in these mice (Table 1 and (1)).

To demonstrate that this defect is not antigen- and adjuvant-specific, we also immunized CRH-tg and WT mice with SRBC, a potent stimulator of germinal center formation in WT mice (Fig. 1 (225, 306)). Again, germinal centers were virtually absent in spleens of CRH-tg mice, while abundantly present in WT mice (Fig. 1).

Splenic architecture is normal in CRH-tg mice

It has been shown recently that impaired germinal center formation may also be associated with abnormal splenic architecture. Mice deficient in lymphotoxin (LT)-α and -β, LTβR or any of several NF-κB subunits all fail to form germinal centers and display abnormal splenic architecture in that T and B cell areas are not segregated and the marginal zone is absent or diminished (145-147, 186, 225, 307). We tested whether T and B cell segregation in the spleen was abnormal in CRH-tg mice by staining with αCD5 (T cells) and αIgM (B cells). The white pulp of both WT and CRH-tg spleens showed a clear demarcation between periarteriolar lymphoid sheath (PALS), comprised primarily of T cells ($CD5^+$, red), and the surrounding B cell follicles (IgM^+, green) (Fig. 2). Additionally, the marginal zone, comprised of a thin layer of metallo-macrophages (MOMA-1, purple) surrounded by marginal zone B cells (green), was normal in both CRH-tg and WT mice (Fig. 2). Although follicles are smaller in size, overall, CRH-tg mice appear to have normal splenic architecture and thus the lack of germinal center formation may reflect a specific defect in the development of appropriate humoral responses to antigen challenge.

Exogenous corticosterone treatment inhibits germinal center formation

We next tested whether elevated corticosterone was sufficient to impair germinal center formation. We treated C57BL/6 WT mice with corticosterone orally for five weeks prior to and one week following immunization with SRBC. Mice were treated with vehicle (0.8% ethanol) or with 30, 60, or 90 ug/ml corticosterone in their drinking water, which increased serum corticosterone levels dose-dependently (Fig. 3). Administration of the two highest doses of corticosterone (60 and 90 ug/ml) achieved levels of circulating corticosterone similar to those in CRH-tg mice (Fig. 3). We

found that corticosterone treatment of WT mice decreased both the number and size of splenic germinal centers (Fig. 4 and 5). This impairment reflects a >10-fold decrease in germinal center area per mm^2 (60 and 90 ug/ml doses, Fig. 5) and an even greater reduction overall given that corticosterone treatment decreased spleen weight by ~50% (data not shown).

This inhibition of the germinal center response, while not complete, has functional consequences. Following immunization, corticosterone-treated mice had α-SRBC IgG titers that were reduced by 6-fold compared with vehicle-treated mice (Table 2). Importantly, levels of α-SRBC IgM were similar between treatment groups; thus, the decrease in IgG indicates that corticosterone treatment impairs isotype switching.

In addition, even short-term treatment with corticosterone impaired germinal center formation. In a separate experiment, mice received corticosterone in their drinking water following, but not prior to, SRBC immunization. While the total number of germinal centers was similar between corticosterone- and vehicle-treated animals, corticosterone treatment following immunization decreased average germinal center size and proportion of the spleen consisting of germinal centers (Table 3). This indicates that corticosterone inhibits germinal center formation via effects that occur both prior to and during an immune response.

Follicular dendritic cell networks are altered by elevated corticosterone

FDC networks are critical to the germinal center reaction (200, 227), and the failure to form germinal centers is associated with loss of FDC in several other systems (145-147, 186, 225, 307). We used the FDC-specific mAb, FDC-M1, to examine FDC networks in corticosterone-treated

mice (191). Vehicle-treated mice showed networks of FDC-M1 staining in the B cell follicles following immunization. In contrast, FDC-M1 staining was severely reduced in intensity and distribution in corticosterone-treated mice (Fig. 6A, top).

As additional markers of FDC networks, we stained spleen sections of immunized mice for complement receptors 1 and 2 (αCD21/CD35) and FcγRII/III (αCD16/CD32), which are expressed at high levels on FDC and at lower levels on B cells (223). Interestingly, while corticosterone treatment drastically reduced the expression of FDC-M1 and FcγRII/III (Fig. 6A, top and middle), complement receptor expression showed a more modest decrease (Fig. 6A, bottom). While more widespread than FDC-M1 or FcγR, expression of complement receptor overlapped with FDC-M1 in vehicle-treated spleens and thus appears to stain an FDC-like network (Fig. 6B). Taken together, these data indicate that corticosterone treatment decreases mature FDC networks.

To determine whether these changes bear functional consequence, we tested the ability of FDC to trap antigen-antibody complexes during an immune response by staining spleen sections with peroxidase-anti-peroxidase immune complexes (PAP) in vitro. Intense follicular PAP staining is seen clearly in vehicle-treated follicles following SRBC immunization (Fig. 7, top left panel). PAP staining co-localized with germinal centers as determined by staining of serial sections with PNA (data not shown). In contrast, spleens from corticosterone-treated, immunized animals showed a marked decrease in follicular PAP staining (Fig. 7, top right panel). This indicates that corticosterone treatment alters antigen-antibody complex trapping ability during an immune response.

Antigen-antibody complex trapping by FDC is normally enhanced during an immune response. Thus, the impaired trapping in corticosterone-treated mice shown above may indicate that FDC are inherently defective or that signals required to enhance antigen-antibody trapping are not generated during an adaptive immune response in these mice. To address whether corticosterone alters immune complex trapping in the absence of an ongoing immune response, we assessed antigen-antibody complex trapping in naive mice *in vivo*. We injected naive control (vehicle-treated) and corticosterone-treated mice with PAP (30 uL, i.v.) and harvested spleens 24 hours later. Spleens from control animals displayed intense networks of staining in the follicles (Fig. 7, bottom far left and left panels) and scattered complexes in the red pulp. In contrast, spleens from corticosterone-treated mice showed a marked reduction in follicular trapping of PAP (Fig. 7, bottom right panel). Both the percent of follicles containing PAP complexes and the number of PAP networks per area of spleen tissue were decreased by corticosterone treatment (Table 4). This finding indicates that corticosterone treatment affects the basal state of FDC networks, independent of effects mediated by activation of the adaptive immune response.

Discussion

We have discovered a novel immuno-modulatory effect of glucocorticoids that leads to marked inhibition of germinal center formation during an immune response. We also show that FDC networks are severely compromised by glucocorticoid treatment, as shown by loss of FDC-specific markers from follicles and impaired ability to trap antigen-antibody complexes—changes which may underlie the glucocorticoid-induced defect in germinal center formation. Impaired FDC-mediated trapping of immune complexes following corticosterone treatment occurred in the absence of an ongoing immune response, which suggests that the defect is inherent to FDC rather

than secondary to a failure to develop the appropriate signals for efficient trapping during an antigen-stimulated immune response.

To our knowledge, this is the first evidence that corticosterone inhibits germinal center formation. In a previous study, a high affinity glucocorticoid receptor (GR) agonist, prednisolone, administered acutely to rabbits at the peak of a primary response did not alter the primary antibody response, but minimized a subsequent secondary antibody response (135). This correlated with deterioration of pre-existing germinal centers due to massive lymphocyte apoptosis (135, 136). The mechanism responsible for this effect is likely different from our results where we administered corticosterone for five weeks prior to immunization. In the current study, we used physiologic levels of corticosterone and found no obvious histologic evidence of apoptosis in follicles, although we have not formally tested this by TUNEL analysis. Rather, we propose that mice treated with corticosterone and the CRH-tg mice have impaired FDC networks, which contribute to a generalized failure to form germinal centers.

FDC are critical elements in germinal center formation (200, 227). Early de novo germinal center formation can be initiated in the absence of FDC; however, complete maturation of germinal centers depends on the presence of FDC (216, 308). FDC provide antigen-independent signals to germinal center B cells that influence proliferation, cell survival, costimulation, and chemotaxis, in addition to providing a source of retained antigen (222, 223, 309, 310). Thus, the disturbed FDC networks we find in corticosterone-treated mice may underlie poor germinal center formation. However, these effects do not preclude the possibility of other disruptions, such as apoptosis of B cells newly entering the germinal center or inadequate T cell help.

Specific mechanisms by which glucocorticoids affect FDC remain to be elucidated. We speculate that corticosterone may inhibit FDC networks and germinal center formation via its known effects on NF-κB mediated gene transcription. Glucocorticoids bind to a cytosolic steroid hormone receptor, GR, which is expressed in most cell types. Once activated by ligand, GR translocates to the nucleus and regulates transcription of numerous genes. A primary mechanism by which GR modulates transcription of immune response genes is through inhibition of NF-κB, which can occur via upregulation of IκB gene transcription, inhibition of IκB degradation, and interference with NF-κB through direct protein-protein interactions (143, 311).

Recent studies have revealed seminal roles for members of the NF-κB family of transcription factors in the formation of germinal centers. Mice with targeted mutations in p52 (146, 147), RelB (145), and bcl-3 (144) all fail to form germinal centers after immunization. The accompanying lack of FDC networks in lymphoid follicles is thought to play a primary role in this defect, and strikingly, proper germinal center formation requires NF-κB expression by stromal cells, but not by hematopoeitic cells (145, 148, 228). This suggests that NF-κB mediated signaling in FDC or FDC precursors may be critical to the germinal center reaction.

Elevated corticosterone, unlike NF-κB deficiency, does not appear to disrupt general splenic architecture (Fig. 2). This suggests that levels of NF-κB may be diminished rather than absent and/or that corticosterone predominantly affects NF-κB mediated gene transcription in FDC/stromal cells rather than hematopoietic cells. Inhibition of NF-κB in FDC or FDC precursors may have several downstream effects consistent with our observations. FDC are defined functionally as cells that trap antigen-antibody complexes (312). We show that this functional ability is decreased severely by corticosterone treatment; however, it appears that immature or less

differentiated FDC may remain because complement receptor staining persists following this treatment (Fig. 6). To maintain their mature phenotype, FDC require tonic stimulation through TNF and LTβ receptors (204, 227, 228, 313, 314), signaling through which is likely to be downregulated via corticosterone-induced NF-κB suppression. Thus, corticosterone may prevent differentiation of precursor FDC or induce de-differentiation of FDC. Furthermore, corticosterone may inhibit FDC function by directly affecting antigen-antibody complex retention. Support for this idea stems from the observation that a single prednisolone treatment can inhibit follicular localization of antibody-opsonized antigen in the spleen without affecting liver retention (315). It is possible that suppression of FcγR expression, which is an effect known to occur with corticosterone treatment (316, 317), contributes to this process in the spleen.

Glucocorticoids are used clinically to treat chronic inflammatory and autoimmune diseases; approximately 30 million Americans suffer from conditions that require long-term treatment with glucocorticoids (318). Our results suggest that antibody responses to immunizations administered concurrently with glucocorticoid therapy may be compromised considerably; these responses would be expected to be short-lived, limited in isotype composition and markedly reduced in affinity. Evidence supports this contention—rheumatic patients and premature infants treated with glucocorticoids develop poor anti-influenza antibody responses compared with patients not treated with steroids (319, 320). Importantly, even a brief treatment of corticosterone given concurrently with immunization impaired germinal center formation, albeit to a lesser degree (Table 3). Thus, even short-term glucocorticoid treatment may pose deleterious consequences to humoral immune responses. Our results also point to a potential target of glucocorticoid therapy in autoimmune states where production of high affinity antibodies that have undergone isotype switching underlies disease pathology. Such patients frequently form extra-lymphoid germinal centers in

affected tissues, and these germinal centers are thought to contribute to autoantibody production (321, 322). Illuminating the mechanism(s) whereby glucocorticoids exert their effect on germinal centers will aid in the identification of more specific targets and lead to better strategies in immunotherapy.

Acknowledgements

We greatly appreciate the immunofluorescent analysis of splenic architecture provided by John Kearney. We wish to thank Marvin B. Rittenberg for insightful discussions, critical review of the manuscript, and T15 monoclonal antibodies. We also thank Greg Wiens and McKay Brown for technical advice, Sarah Coste for editorial expertise, and Marie Kosco-Vilbois for FDC-M1 monoclonal antibody used in pilot experiments.

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Table 1. *CRH-tg mice generate markedly reduced anti-PC IgG responses with altered specificity*

Days[a]	αPC-protein IgG (ug/mL)		% inhibition by free PC	
	WT	CRH-tg	WT	CRH-tg
7[b]	1.69±0.3[c]	0.73±0.25*	88±3.2	79±7.5
19[b]	1338±261	145±25.2*	45±12	94±3.7*
26[b]	1981±357	227±46.5*	49+13	92±4.7*
35	2559±493	247±26.8*	53±14	95±3.5*
50	1789±452	155±39.1*	49±13	93±4.1*

[a] Mice were immunized i.p. with 70 ug PC-KLH in CFA at day 0, then boosted i.p. with 70 ug PC-KLH in IFA on day 14.

[b] These timepoints were published previously.

[c] Results are mean ± SEM of 8 mice per group.

*$p<0.01$ significantly different from WT by one-way ANOVA, Student-Neuman-Keuls post hoc analysis.

Table 2. *Corticosterone treatment inhibits isotype switching after SRBC immunization*[a]

	IgM titer	IgG titer
Fold difference[b]	1.2 ± 0.25	5.8 ± 0.54*

[a] Mice were treated orally with vehicle or corticosterone (90 ug/mL) for 35 days prior to i.p. SRBC immunization; day 7 post-immunization titers are shown.

[b] Data are expressed as the mean fold difference between paired samples of vehicle and corticosterone-treated mice (± SEM, n=8 pairs).

*$p<0.01$, Student's *t* test for paired sample means.

Table 3. *Corticosterone administered concurrent with immunization reduces germinal center size*

	Vehicle	Corticosterone[a]
Germ. ctr. size[b]	0.0855 ± 0.001	0.053 ± 0.0002*
# of germ. ctr./mm^2	0.231 ± 0.017	0.186 ± 0.007
% germ. ctr. area/spleen area	1.824 ± 0.62	1.056 ± 0.471*

[a] Mice were treated with 60 ug/mL corticosterone or vehicle alone in their drinking water starting at the time of i.p. SRBC immunization and continuing until tissue harvest 7 days later.

[b] Data are expressed as the mean splenic germinal center size in mm^2 (± SEM, n=7 per group).

*$p<0.01$, Student's *t* test for paired sample means.

Table 4. *Exogenous treatment with corticosterone decreases in vivo trapping of antigen-antibody complexes (PAP)*

	Vehicle	Corticosterone[a]
% of follicles with PAP networks[b]	49.8 ± 4.4[c]	9.2± 2.9*
# of PAP networks/mm^2	1.8 ± 0.18	0.38 ± 0.15*

[a] Mice were treated with vehicle or 60 ug/ml corticosterone for 4 weeks, then injected i.v. with 30 uL PAP. Twenty-four hours later, spleens were harvested.

[b] Peroxidase activity was developed, and networks were enumerated by NIH Image analysis on scanned images.

[c] Results are mean ± SEM of 6-8 mice per group.

*$p<0.05$ vs. vehicle-treated, Student's *t* test.

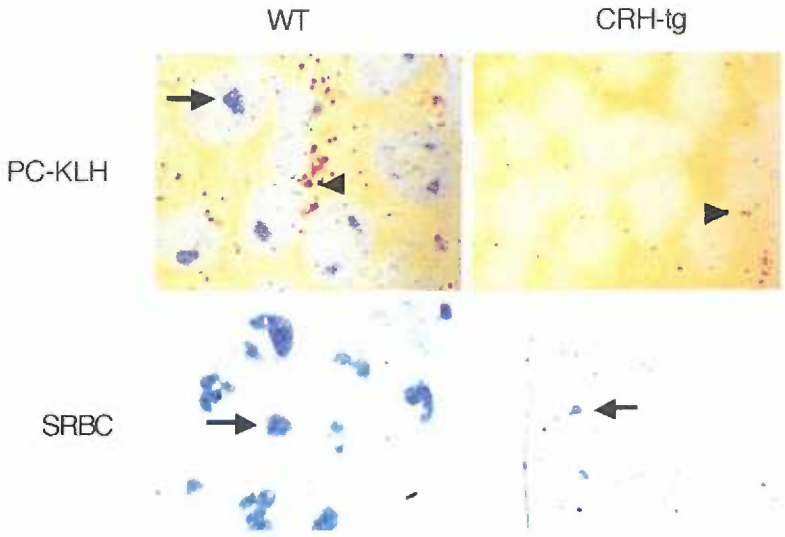

Figure 3-1. CRH-tg mice fail to form germinal centers after immunization. Mice were immunized i.p. with 70 ug PC-KLH in CFA (top panels) or 200 uL SRBC diluted 1:10 in HBSS (bottom panels) and spleens collected seven days later. Frozen sections were stained with PNA (blue, all panels) and AB1-2 (red, top panels only). Arrows denote germinal centers; arrow heads denote foci of AB1-2 stained plasma cells. Micrographs are representative of 7-8 spleens per genotype. Original magnification, 5x.

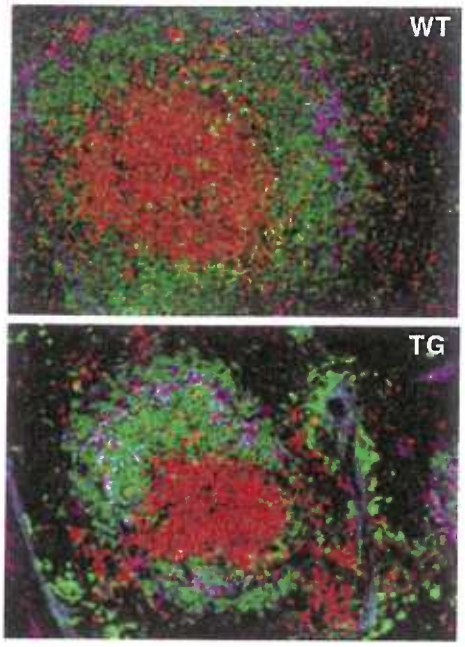

Figure 3-2. CRH-tg mice have normal splenic architecture. Frozen spleen sections from naive mice were stained with αCD5 (T cells, red), αIgM (B cells, green), and MOMA-1 (metallomacrophages, purple) and analyzed by immunofluorescence microscopy. One representative area of white pulp is shown in each panel. Original magnification, 100x.

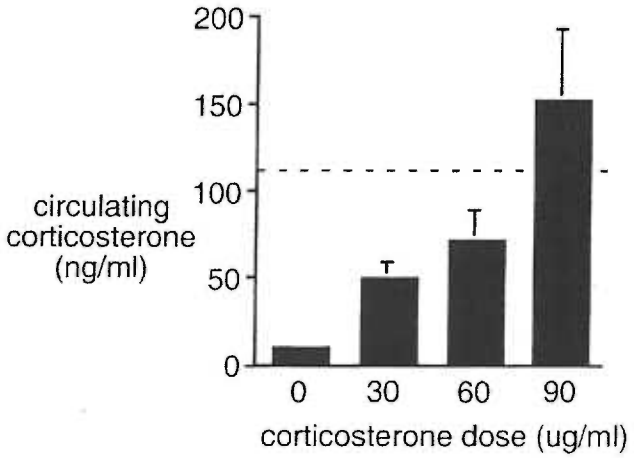

Figure 3-3. Corticosterone in drinking water results in a dose-dependent increase in circulating corticosterone levels (significant effect of dose; $p < 0.05$, one-way ANOVA). Mice were treated for five weeks with vehicle (0.8% ethanol) or various doses of corticosterone. Basal (non-stress, a.m.) plasma samples were assayed for corticosterone by commercial RIA. Dashed line indicates typical CRH-tg level of circulating corticosterone. Results are the mean ± SEM of 8-10 mice per group.

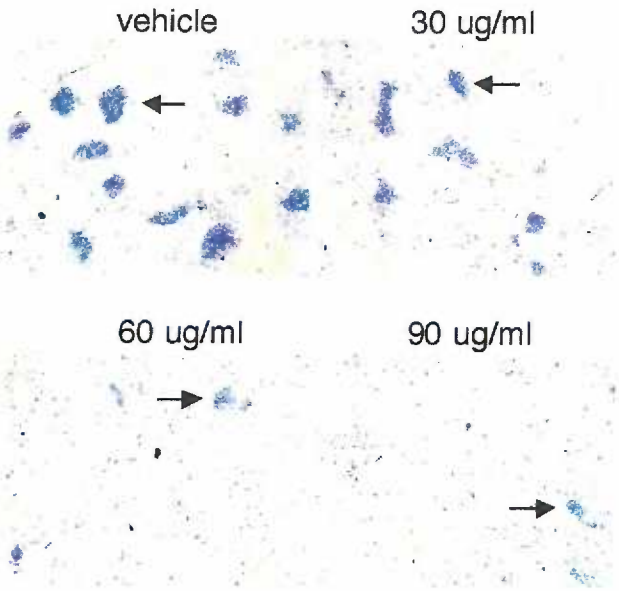

Figure 3-4. Corticosterone treatment causes a dose-dependent decrease in germinal center formation. Mice were treated orally with vehicle or the indicated doses of corticosterone for five weeks, followed by i.p. immunization with 200 uL SRBC diluted 1:10 in HBSS. At day seven after immunization spleens were collected and frozen sections were stained with PNA (blue). Photomicrographs are representative of 6-8 spleens per group. Arrows denote representative germinal centers. Original magnification, 5x.

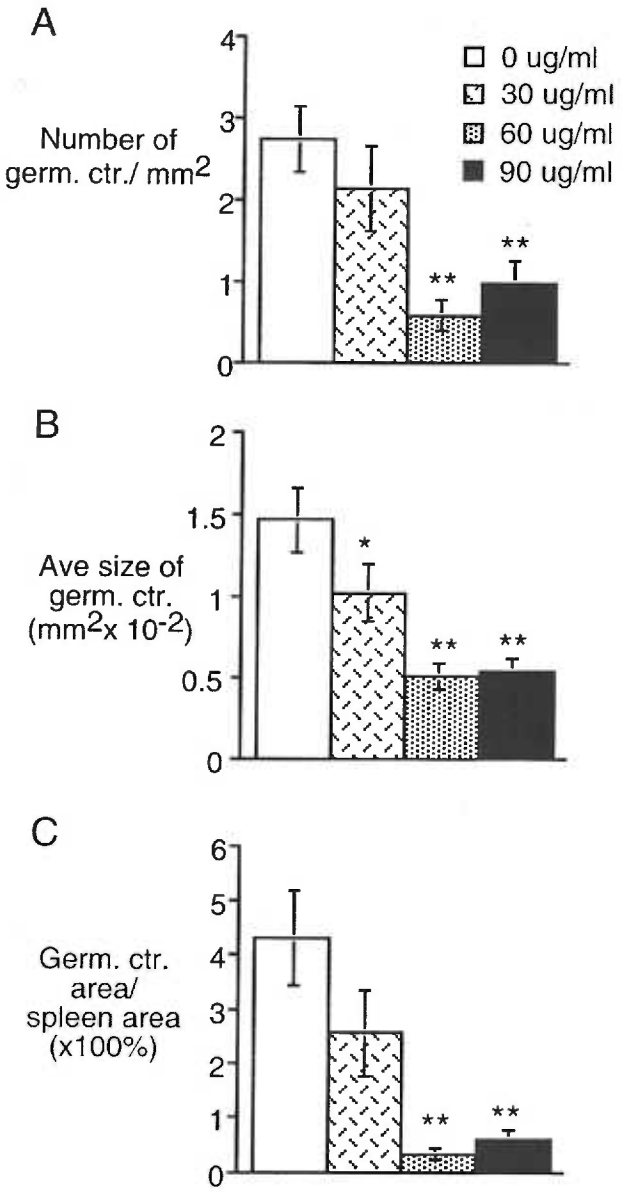

Figure 3-5. Corticosterone decreases germinal center number and size. Mice were treated orally with vehicle or corticosterone for five weeks and then immunized i.p. with 200 uL SRBC (diluted 1:10 in HBSS). Spleens were processed at day seven and stained as in figure 4. Germinal centers were enumerated and measured using NIH Image software analysis on scanned images. Note that all calculations are normalized to spleen area. *A*, average number of germinal centers per mm2 of spleen tissue. *B*, average germinal center size. *C*, percent of the spleen area that is encompassed by germinal centers. Data represent the mean ± SEM of 6-8 spleens per group. *A-C*, $p < 0.05$ significant effect of dose on germinal centers, one-way ANOVA. *, $p < 0.05$ different from vehicle, Student-Newman-Keuls post-hoc analysis. **, $p < 0.05$ different from vehicle and 30 ug/mL dose, Student-Newman-Keuls post-hoc analysis.

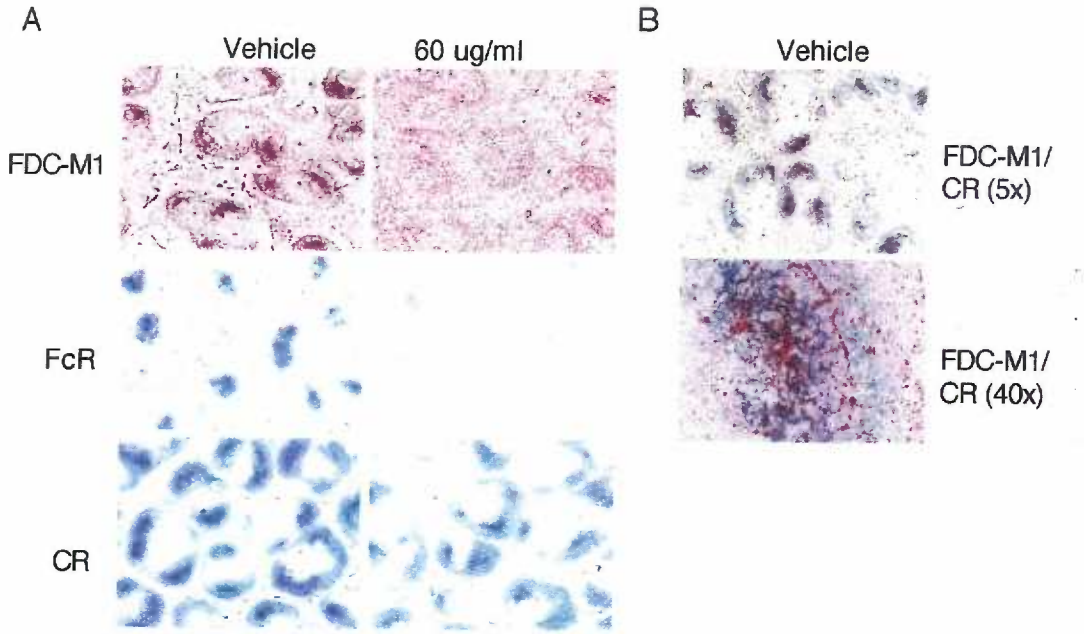

Figure 3-6. Corticosterone treatment diminishes FDC networks. Mice were treated orally with vehicle or with 60 ug/mL corticosterone for five weeks, then immunized with SRBC. Seven days later, spleens were frozen and tissue sections were stained with: *A*, FDC-M1, αCD16/CD32 (FcγRII/III, FcR) or αCD21/CD35 (complement receptors 1 and 2, CR) as indicated. *B*, both FDC-M1 (red) and αCR (blue). Images are representative of 6-8 mice per treatment. Original magnification, 5x unless otherwise indicated.

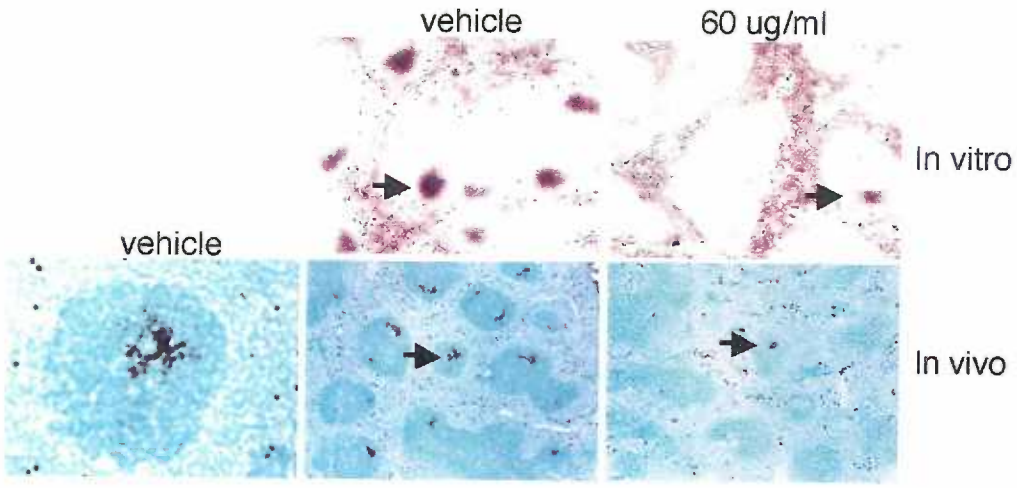

Figure 3-7. Corticosterone treatment inhibits antigen-antibody complex trapping in vitro and in vivo. Mice were treated orally with vehicle or 60 ug/mL corticosterone for 4-5 weeks. *Top panels*, mice were then immunized with 200 uL SRBC (diluted 1:10 in HBSS) and seven days later spleens were collected and frozen sections were stained with PAP (brown). *Bottom panels*, mice were injected i.v. with 30 uL PAP; 24 hours later spleens were collected and frozen sections were developed for peroxidase activity (red/brown) and counter stained with methyl green. Images are representative of 5-7 spleens per group. Arrows indicate PAP staining. Original magnification, 5x (middle and right panels) or 40x (far left panel).

Chapter 4—Manuscript # 3

Chronic environmental stress does not affect germinal center formation, despite elevations in circulating corticosterone

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Abstract

Activation of stress responsive pathways can affect subsequent immune responses. One well-studied mechanism by which this occurs is activation of the HPA axis. Humoral immune responses to thymus-dependent antigens are diminished in many models of environmental stress, and exogenous glucocorticoids have been shown to exert a similar effect. We showed previously that chronic HPA activation in CRH transgenic mice leads to diminished antibody responses, poor isotype switching, and poor memory. We determined that inhibition of germinal center formation underlies these defects and that elevated corticosterone was sufficient to mediate these effects. Here, we investigate the ability of chronic predator and chronic restraint stress to impair germinal center formation. Despite robust and prolonged HPA activation, neither stress altered germinal center formation in response to SRBC immunization. We speculate that glucocorticoid elevations must be continuous in order to affect germinal centers or that additional pathways activated during chronic stress can counteract deleterious effects of HPA activation on germinal center formation.

Introduction

Stress has long been associated with increased susceptibility to certain diseases, which include infection, metastases, and autoimmunity. In addition, stress can significantly alter numerous immune parameters. This has led to the generally accepted idea that increased susceptibility to infectious pathogens and cancer on the one hand, and increased autoimmunity on the other hand, results from stress-induced dysregulation of immune function.

Stress-induced suppression of cellular immunity is well documented in both humans and animal models of stress and can result from decreases in cell number as well as functional inhibition. Both acute (minutes to hours) and chronic (days to weeks) stress in a variety of models inhibit T cell and NK cell-mediated cytotoxicity (102-104, 323-328). These changes likely bear physiologic relevance as they correlate with disease severity in models of influenza and HSV infection (102, 327, 328).

The effect of stress on humoral immunity is less well-established. Some confusion on this topic stems from differences between pre-immune (non-specific and/or natural) antibodies and antigen-specific responses. Total non-specific antibody titers are usually either increased or unchanged by stress (329-332). Frequently, this is used as an index of humoral immunity in human studies, which leads to the conclusion that humoral immunity is unaffected. In contrast, stress generally depresses antigen-specific antibody responses (288, 333-340). Notably, this is specific for thymus-dependent responses, whereas thymus-independent responses to non-protein antigens are usually unaffected (288, 338, 341-343). In addition, stress inhibits IgG responses more severely than IgM

responses (335, 336, 339, 340, 344). Thus, there is a qualitative aspect to stress-induced inhibition of antibody responses, with TD responses and IgG isotypes showing greater sensitivity.

HPA activation is one well-studied pathway by which stress modulates immune responses, and exogenous glucocorticoid administration potently inhibits antibody responses. While effects of exogenous glucocorticoids frequently are more severe than environmental stress, the pattern is similar in that TD, but not TI, and IgG, but not IgM, responses are affected (126, 129, 130, 345). We showed previously that chronic HPA overactivation in CRH-tg mice also inhibited IgG responses to TD antigens to a greater degree than IgM (1). Moreover, WT mice treated orally with corticosterone recapitulate this effect on antibody responses.

To identify immunologic targets contributing to the humoral defect in CRH-tg and corticosterone-treated WT mice, we examined germinal center formation and found that these structures were nearly abolished by chronic HPA activation. Germinal centers provide a critical microenvironment for the maturation of TD humoral responses because they are the site of affinity maturation, isotype switching, and memory B cell formation. In contrast, TI responses do not elicit robust germinal center formation and are not characterized by an isotype switch or memory B cell development. Thus, we believe that defects in germinal center formation underlie the suppression of isotype switching induced by chronic HPA activation.

Robust HPA activation is a hallmark of stress. Thus, we hypothesized that chronic environmental stress may also negatively impact germinal center formation, and that this mechanism might explain why stress preferentially suppresses TD and IgG responses, while exerting a lesser effect on TI and IgM responses. Here, we test this hypothesis in two models of chronic stress, predator

stress and restraint stress. In both cases mice were exposed to the stressor for several weeks prior to immunization and application of the stressor continued until the day of sacrifice 7 or 13 days following stress. We found that, despite robust elevations in circulating corticosterone similar to those observed in CRH-tg and corticosterone-treated mice, neither of these environmental stressors inhibited germinal center formation.

Methods

Mice

C57BL/6 12 week old male mice were used in all experiments. Mice were group-housed under SPF conditions on a 12 hour light-dark cycle at Oregon Health and Science University and given free access to food and water except during the stress procedure. Control, non-stressed mice were deprived of food and water for the same duration each day. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University.

Predator stress

Mice were exposed to one singly-housed male Long-Evans rat (~300 g) for 1.5 hours per day between 08:00 and 12:00 (light cycle). Immediately prior to stress mice were transferred to a new clear plastic box with wire top. This box was then placed inside the rat cage. Thus, the mouse was exposed to the sight and smell of the rat without physical contact. Control mice remained in their home cages.

Rats would frequently sit on top of the mouse cage and move the mouse cage. Twelve different rats were employed; thus, mice were rotated among the rats on different days to avoid habituation by either "predator" or "prey." Each rat acted as stressor for two mice, consecutively, each day. Mice alternated daily between being the first or second "prey" of the day to control for rats becoming disinterested by the second subject. Stress was repeated daily for a total of 21 or 27 days. At day 14, 16 mice from each group (stressed and non-stressed) were immunized with SRBC; three additional mice from each group served as saline-immunized controls. Spleens were harvested from eight SRBC-immunized mice from each group at day 21 (day 7 after immunization); the remaining SRBC-immunized and saline control spleens were harvested at day 27 (day 13 after immunization).

Restraint stress

Mice were restrained in 50 ml conical polypropylene tubes equipped with air holes for 9 hours per day from 08:00 to 05:00 (light cycle). During this time food and water was not available. Mice were kept warm with an indirect heat lamp to avoid hypothermia. Control mice remained in their home cages, but were deprived of food and water for this period. Within the tubes, mice could roll right and left, but could not turn around head to tail. Stress was repeated daily for a total of 42 or 48 days. At day 35, 16 mice from each group (stressed and non-stressed) were immunized with SRBC; three additional mice from each group served as saline-immunized controls. Spleens were harvested from eight SRBC-immunized mice from each group at day 42 (day 7 after immunization); the remaining SRBC-immunized and saline control spleens were harvested at day 48 (day 13 after immunization).

Immunization and tissue collection

Mice were immunized i.p. with 200 uL defibrinated SRBC (Colorado Serum Co., Denver, CO) diluted 1:10 in sterile, endotoxin-free HBSS. Control mice received 200 uL HBSS i.p. Seven or 13 days after immunization spleens were harvested and placed in cryomolds in cold OCT and frozen in isopentane chilled in liquid nitrogen. The blocks were stored at –80°C until sectioning.

Corticosterone measurement

Blood for plasma corticosterone measurement was obtained from the retro-orbital plexus and collected in 40 uL EDTA (7.5 mg/ml). Mice were bled before and after stress on the indicated days within 90 seconds of disturbing the cage in order to minimize any unintended environmental stress. No mouse was bled more than once every three days. Cells were pelleted and the remaining plasma was stored at –20°C until assay by commercial RIA (ICN, Costa Mesa, CA) according to the manufacturer's instructions.

Immunohistochemistry

Frozen sections of spleens were cut (10 um) onto poly-L-lysine treated slides, fixed in cold acetone, and stored at –80°C. For colormetric detection, tissue sections were blocked for 30 min in TNB blocking buffer (0.1 M Tris-HCl, 0.15 M NaCl, 5 mg/ml blocking reagent (tyramide amplification kit, Perkin Elmer Life Sciences, Boston, MA)). Sections were then incubated for one hour with peanut agglutinin (PNA, Vector, Burlingame, CA), washed 3x in wash buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.1% Tween-20), then incubated with goat-anti-PNA secondary for one

hour (Vector), washed as before, and finally incubated for one hour with an AP-conjugated rabbit-anti-goat tertiary antibody (Sigma, St. Louis MO). After a final wash, AP enzymatic activity was visualized with 3-amino-ethyl-carbazole (Sigma) and naphthol AS-MX phosphate (Sigma), respectively.

Germinal center and PAP quantitation

Slides were scanned into digital format using a SlideScan 35 (Polaroid, Cambridge, MA). Number and size of germinal centers were calculated using NIH Image software (version 1.61).

Results

Chronic predator stress activates the HPA axis

We have found that both overexpression of a CRH transgene and chronic exogenous administration of corticosterone inhibit germinal center formation. Therefore, we hypothesized that chronic stress, which activates the HPA axis and other CRH-responsive pathways, might also affect germinal center development. Our first approach was to use a model of psychological stress, chronic predator stress. We used male Long-Evans rats as the "predator" and allowed mice to see, hear, and smell, but not interact physically with rats. This has been shown previously to represent a potent, ethologically relevant stressor (346-348).

As expected, predator stress induced a robust increase in circulating corticosterone (Fig. 1). Mice were bled retro-orbitally immediately before and after stress every fourth day of stress. Post-stress corticosterone levels were significantly higher than pre-stress levels at all time points and mirrored

levels typically seen in CRH-tg mice (1). This response did not habituate over the course of the experiment; in fact, one-way ANOVA showed a significant increase in post-stress corticosterone over time. However, basal (pre-stress) levels of corticosterone did not increase over the course of the experiment. Thus, repeated predator stress caused chronic stress-induced elevations in corticosterone, but did not alter basal hormone levels.

Chronic predator stress does not alter germinal center formation

We immunized mice on the 14th day of predator stress, and continued daily stress until the time of tissue harvest 7 or 13 days later. We stained spleen sections with peanut agglutinin (PNA), a lectin that binds specifically to germinal center B cells. PNA staining looked grossly similar between control and predator stressed mice at both time points (Fig. 2). We quantitated the number and average size of germinal centers on a per area basis and found no difference between control and stressed mice at either time point (Fig. 3). In both groups, unimmunized controls had few to no splenic germinal centers. Thus, chronic predator stress does not alter the overall kinetics or magnitude of the germinal center response.

Chronic restraint stress activates the HPA axis

Various types of stress can activate different stress-responsive pathways, leading to potentially different physiological outcomes (349). In addition, it is possible that the daily duration and number of days that mice were exposed to predator stress were insufficient to affect germinal centers since CRH-tg and corticosterone-treated mice have elevated corticosterone constitutively. Thus, we employed a second model of chronic stress, repeated restraint stress. This is considered a

combined physical/psychological stress (81), and we subjected mice to nine hours per day of stress for five weeks prior to immunization.

Similar to chronic predator stress, restraint stress induced a robust increase in corticosterone. Corticosterone elevation was maintained for the entire nine hours, and again mice showed little evidence of habituation over the course of the experiment (Fig. 4). While basal (a.m.) levels of corticosterone did rise slightly over the course of the experiment, this was not different between control and stressed mice, and likely represents a learned anticipation of handling and retro-orbital bleeding. Furthermore, basal levels of corticosterone in both groups remained within a normal non-stressed range. Thus, restraint stress caused a robust increase in circulating corticosterone for at least nine hours per day for five weeks.

Chronic restraint stress does not alter germinal center formation

We immunized mice on the 35th day of restraint stress, and continued restraint stress until the time of tissue harvest 7 or 13 days later. Similar to predator stress, restraint stress did not appear to have any obvious qualitative or kinetic effect on germinal center formation (Fig. 5). Quantitation confirmed that the number and size of germinal centers was not different between control and stressed mice at either time point (Fig. 6). Thus, like predator stress, restraint does not alter the ability of mice to form germinal centers in response to immunization.

Discussion

We have shown here that neither chronic predator stress nor chronic restraint stress alters germinal center formation following SRBC immunization, despite robust increases in corticosterone. Several explanations could account for the discrepancy we see between immune effects of chronic HPA activation and corticosterone elevations and effects of chronic stress. It is possible that other pathways (autonomic, peptidergic, etc.) activated during the stress response may oppose the immunosuppressive effect of glucocorticoids on germinal center formation. Such compensation has been reported during stress wherein epinephrine exerts pro-inflammatory effects that counterbalance the anti-inflammatory effects of glucocorticoids (350), and studies have indicated that endogenous opioids produced during stress may enhance antibody production (351, 352). This may be an adaptive feature designed to allow maintenance of certain immune responses during times of prolonged stress and high glucocorticoid levels. Alternatively, continuous elevations in corticosterone may be required to influence germinal centers. During chronic repeated stress, corticosterone is elevated throughout each stress treatment, but returns to baseline during stress-free intervals between treatments. In contrast, CRH-tg and corticosterone-treated mice maintain elevated corticosterone levels—a feature that may be necessary to significantly affect germinal center formation.

The question emerges, if chronic stress does not alter germinal centers, why does impaired switching to IgG occur? It is possible that despite normal appearance of germinal centers, function may be compromised during chronic stress. Thus, there may be a difference between the threshold for visible germinal center formation and the threshold for isotype switching. Germinal centers are defined histologically as clusters of PNA positive B cells that form in the follicles of lymphoid

tissues ~5 days following antigenic challenge. Numerous signals from T cells and follicular dendritic cells (FDC) are required for germinal center formation and for consequent maturation of the humoral response to proceed. These signals, which include pro-inflammatory cytokines, costimulatory molecules, transcription factors, and complement pathway components, have been elucidated primarily by means of genetic knock-out studies in mice (170, 183-187, 218-220, 225). Deletion of many of these genes abolishes germinal centers as well as isotype switching, affinity maturation, and memory cell formation. However, the effect of more subtle impairments in any of these signals on germinal center formation and maturation of the humoral response is unclear. For instance, activation of stress-responsive pathways may not hinder T cells from providing adequate help to activate B cells to move into follicles, cluster around FDC networks in the follicles, and express the PNA ligand, but may inhibit additional signals, such as T cell-derived cytokines that are necessary for isotype switching.

An alternative explanation is that stress has deleterious effects on IgG following productive isotype switch in germinal centers. For instance, individual B cell clones may undergo isotype switching and affinity maturation, but fail to survive/proliferate or fail to secrete immunoglobulin following emergence from the germinal center.

What are the consequences of decreased IgG production to host defense? IgG has a significantly longer half-life than IgM and possesses effector functions that IgM does not, including opsonization of pathogens for Fc receptor-mediated recognition by phagocytes and sensitization of cells for antibody-dependent cell-mediated cytotoxicity. Therefore, decreased IgG may predict a less favorable outcome in the face of infection. While antibody titers elicited by high doses of antigen coupled with proinflammatory adjuvants may not be limiting, decreased isotype switching

is usually associated with impaired memory B cell generation and affinity maturation. Thus, during stress the quantity and quality of long-term memory after immunization may be hindered sufficiently to increase disease susceptibility, despite normal germinal center appearance. In addition, environmental stress may inhibit germinal center formation in situations where antigen dose or immune stimulation is limited. For example, stress may allow adequate maturation of the humoral immune response after an optimized immunization, but may exert a more deleterious effect on the humoral response to certain types of slow-replicating or poorly immunogenic pathogens.

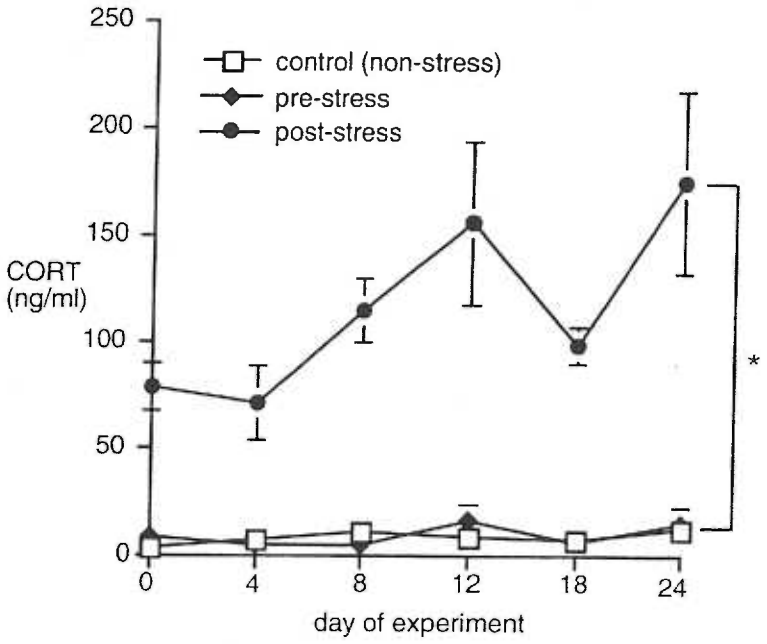

Figure 4-1. Chronic predator stress increases corticosterone during stress, but does not affect basal levels of corticosterone. Control (non-stress) mice and mice exposed to chronic predator stress were bled between 08:00 and 10:00 a.m. on the days indicated. Mice exposed to predator stress were also bled immediately following 1.5 hours predator stress on the days indicated. Serum was frozen for subsequent corticosterone analysis by commercial RIA. Results are the mean ± SEM of 8 mice per group. *$p<0.001$ main effect of group, post-stress different from pre-stress and control.

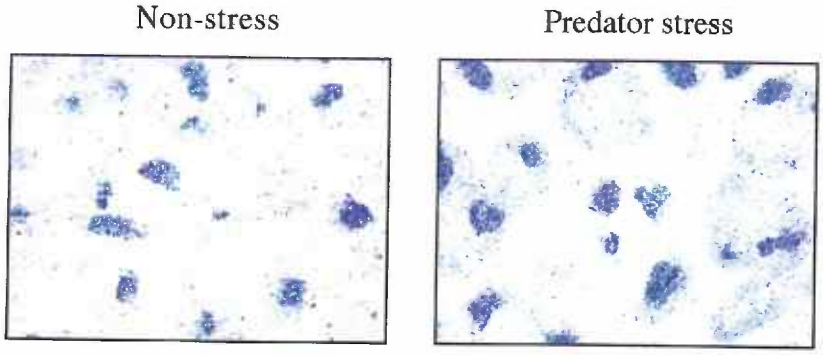

Figure 4-2. Chronic predator stress does not alter germinal center formation. Control mice or mice exposed to chronic predator stress were immunized i.p. with SRBC. Seven days later spleens were harvested and germinal centers stained with PNA. Micrographs are representative of 8 spleens per group.

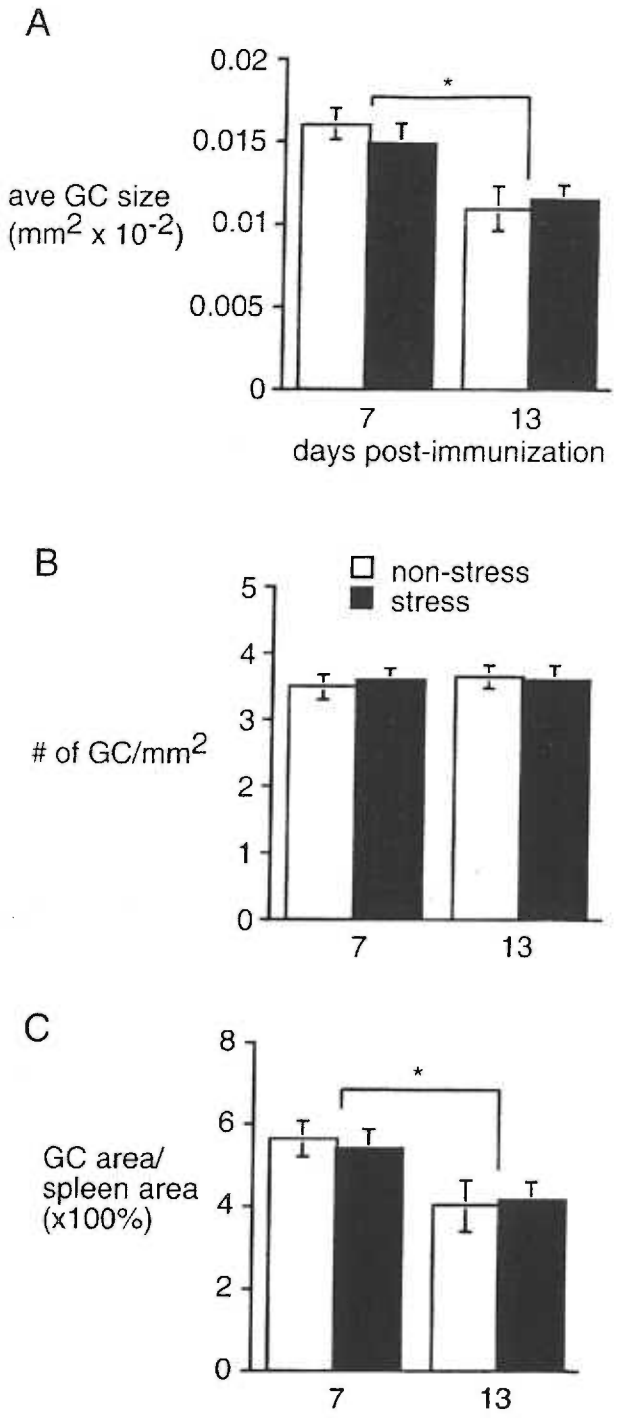

Figure 4-3. Chronic predator stress does not alter kinetics or magnitude of the germinal center response. Control (non-stress) mice or mice exposed to chronic predator stress were immunized and spleens harvested 7 or 13 days later. Germinal centers were enumerated and measured using NIH Image software analysis on scanned images. Note that all calculations are normalized to spleen area. A, average number of germinal centers per mm2 of spleen tissue. B, average germinal center size. C, percent of the spleen area that is encompassed by germinal centers. Data represent the mean ± SEM of 8 spleens per group per time point with the exception of non-stress day 13, n=3. * $p<0.05$ significant main effect of time, one-way ANOVA.

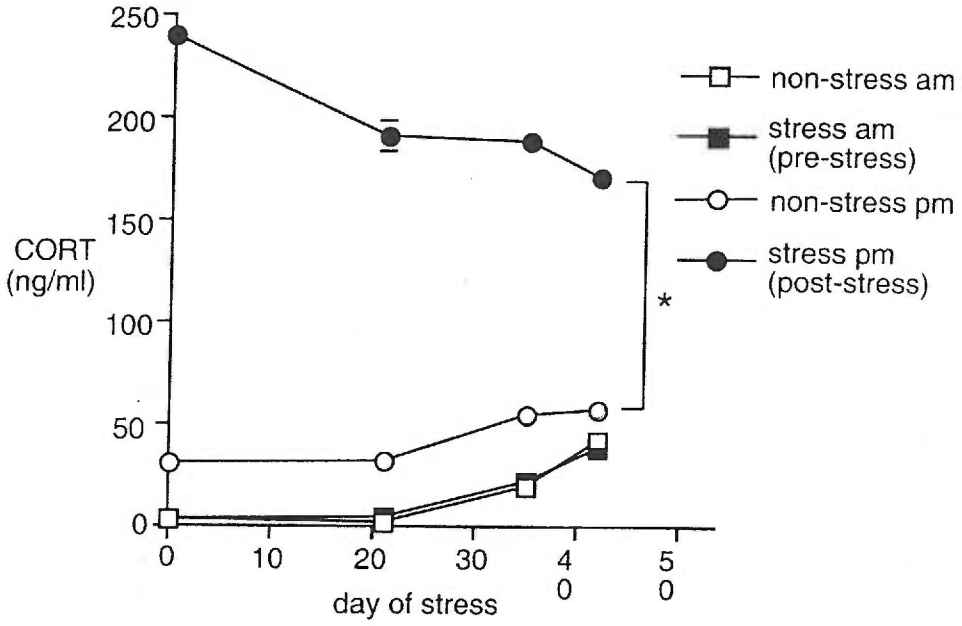

Figure 4-4. Chronic restraint stress increases corticosterone during stress, but does not affect basal levels of corticosterone. Control (non-stress) mice or mice exposed to chronic restraint stress were bled at 8:00 a.m. (pre-stress) and at 5:00 p.m. (immediately post-stress) on the days indicated. Serum was frozen for subsequent corticosterone analysis by commercial RIA. Results are the mean ± SEM of 8 mice per group. *$p<0.001$ main effect of group, p.m. stress different from p.m. non-stress.

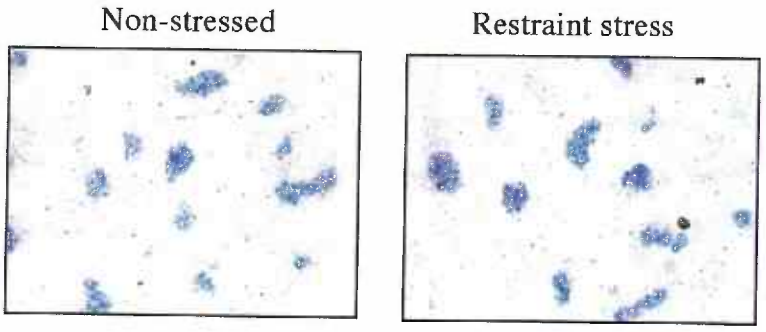

Figure 4-5. Chronic restraint stress does not alter germinal center formation. Control mice or mice exposed to chronic restraint stress were immunized i.p. with SRBC. Seven days later spleens were harvested and germinal centers stained with PNA. Micrographs are representative of 8 spleens per group.

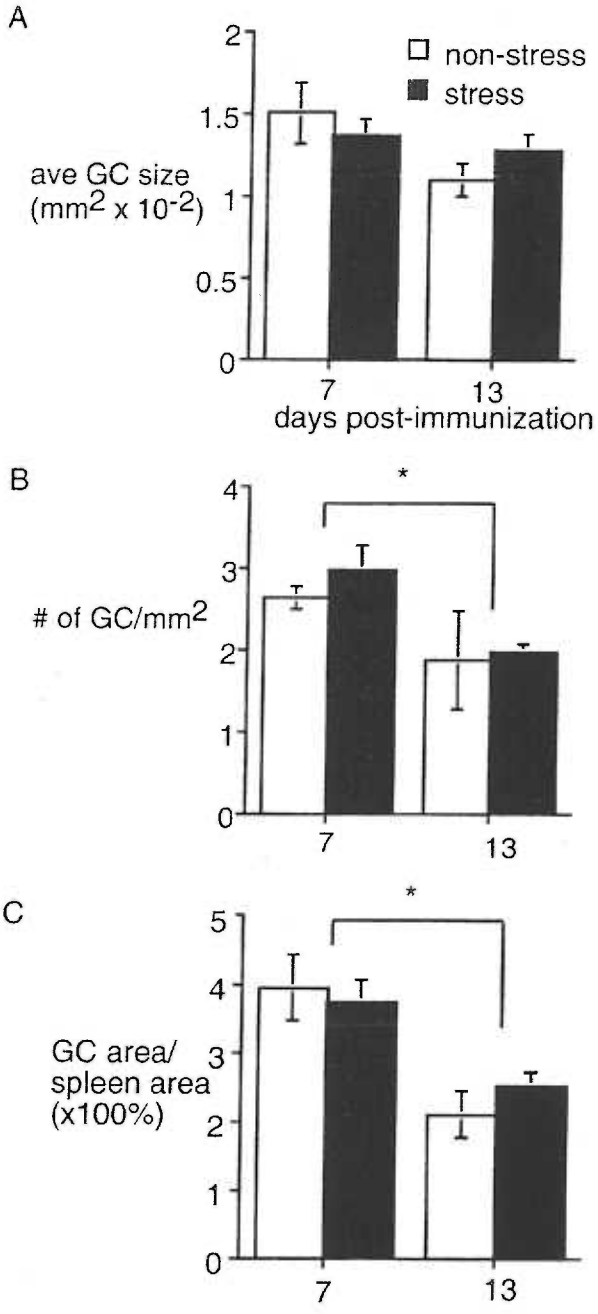

Figure 4-6. Chronic restraint stress does not alter the kinetic or magnitude of the germinal center response. Control (non-stress) mice or mice exposed to chronic restraint stress were immunized and spleens harvested 7 or 13 days later. Germinal centers were enumerated and measured using NIH Image software analysis on scanned images. Note that all calculations are normalized to spleen area. A, average number of germinal centers per mm of spleen tissue. B, average germinal center size. C, percent opf the spleen area that is encompassed by germinal centers. Data represent the mean ± SEM of 8 spleens per group per time point. * $p<0.05$ significant main effect of time, one-way ANOVA.

Chapter 5—Summary and Conclusions

I began this work with the hypothesis that chronic CRH upregulation impairs humoral immunity. I showed this to be true and provide several mechanisms that may underlie this impairment.

B lymphopoeisis

First, I showed that CRH overexpression in CRH-tg mice leads to impaired B cell lymphopoeisis and, subsequently, depletion of mature B cell numbers in the periphery. Total B cell numbers are decreased in the bone marrow. I examined the proportion of B cells in each developmental stage and found that pre B cells were preferentially susceptible. This is likely due to glucocorticoid-induced apoptosis because 1) adrenalectomy of CRH-tg mice reverses B cell loss in both the bone marrow and periphery (238), 2) glucocorticoids induce apoptosis of pre B cells in vitro (160), and 3) exogenous glucocorticoids reduce B cell populations in the bone marrow, with predominant effects on pre B cells (112, 113, 353).

The susceptibility of pre B cells to glucocorticoids is likely related to the expression levels of pro- and anti-apoptotic molecules. Pre and immature B cells have high ratios of the pro-apoptotic bax to anti-apoptotic bcl-2, and this is thought to provide the basis for negative selection of B cells with unproductive rearrangements (163). Thus, pre and immature B cells are programmed to die unless they receive survival signals (which among other effects, upregulate bcl-2) via appropriately assembled antigen receptors. Elevated levels of glucocorticoids may tip this balance in favor of apoptosis even in the presence of BCR signals, since antigen receptors signal, in part, via transcription factors such as NF-κB and AP-1 that are regulated negatively by glucocorticoids

(151, 154, 155, 354-356). Furthermore, glucocorticoid-induced apoptosis correlates with repression of these transcription factors in an analogous stage of T cell development, DP T cells (150, 166).

Aside from mechanism, is there a teleological explanation for the sensitivity of pre B cells to elevated glucocorticoids? The answer here is more speculative, but again, an analogy with developing thymocytes may provide some clues. Glucocorticoids produced locally by thymic epithelium are important in positive selection of thymocytes. In their absence, TCR signals generated through low to moderate avidity antigen receptors (the range of avidity that would normally result in positive selection) induce apoptosis of DP T cells (267, 357). Thus, glucocorticoids may *dampen* the ability of TCR signaling to induce apoptosis and thereby allow survival of T cells with the low self-reactivity necessary to recognize self-MHC. However, at elevated levels of glucocorticoids, this balance may be tipped so that inadequate survival signals are provided by TCR ligation of any avidity, and massive DP T cell apoptosis ensues.

Endogenous levels of glucocorticoids may be important in B cell selection as well. While it is likely that an extracellular preBCR ligand is unnecessary for positive selection of pre B cells, signaling through an assembled preBCR is necessary (358, 359). Similarly, in immature B cells, the newly rearranged light chain must pair with the heavy chain to produce a signaling competent BCR in order for B cell development to proceed. In contrast, signals generated through autoreactive antigen receptors signal B cells to edit or die. Endogenous basal levels of glucocorticoids may be important in regulating this balance between survival of B cells that receive weak signals generated by appropriately assembled antigen receptors and deletion of strongly autoreactive cells. To my knowledge, a role for endogenous glucocorticoids in B cell

selection is untested, but some indirect evidence supports this possibility. First, I have found that CRH-tg mice respond to PC-KLH immunization with an altered antibody repertoire. Although this may simply reflect differential responsiveness upon antigen challenge, I have also observed an increase in T15+ splenic B cells in naïve CRH-tg versus WT mice, suggesting the pre-immune repertoire is different (unpublished observation). Furthermore, in avian species, not only the thymus, but also the bursa, is capable of producing glucocorticoids (360). Together, these data may warrant investigation of a role for glucocorticoids in B cell selection and repertoire.

Antigen-specific responses

I also discovered that CRH overproduction impairs antibody responses following immunization with thymus-dependent antigens. This is consistent with a decrease in peripheral B cells as well as the overall decrease in size of secondary lymphoid organs. However, I also found unexpected qualitative differences. First, the isotype profile differs—while IgM titers are marginally diminished in CRH-tg mice, IgG titers following secondary boost are suppressed profoundly. This indicates that isotype switching is impaired in CRH-tg mice, a conclusion further supported by earlier studies that showed CRH transgene expression causes a greater decrement in IgG PFC elicited during the memory response than in IgM PFC elicited during the primary response (229). In addition to isotype, the fine specificity of antibodies elicited in CRH-tg mice is abnormal. Unlike WT mice, after secondary boost CRH-tg mice do not switch to production of group II antibodies, but rather continue producing antibodies that recognize free PC. The switch to group II antibodies in WT mice is thought to result from expansion of rare precursors that eventually outcompete the initially more ubiquitous T15 idiotype (361). This may be related to the fact that

group II antibodies have a higher affinity for PC-protein and can further increase this affinity via SHM, while T15 does not appear capable of further improving its affinity for ligand (362-364).

These observations that CRH overexpression impairs 1) isotype switching, 2) memory responses, and possibly 3) competition led me to investigate germinal center formation in these mice. I found that, indeed, CRH-tg mice have a near complete inability to form germinal centers. This finding may be a common link underlying the observed humoral defects.

I am interested in the mechanism of these effects from the perspective of stress physiology. Glucocorticoids inhibit IgG more than IgM and impair TD responses while sparing TI (288, 335, 336, 338-344); therefore, I hypothesized that the increase in circulating glucocorticoids in CRH-tg mice is responsible for depressed isotype switching and lack of germinal centers. I treated WT mice with doses of corticosterone that mimic stress levels and found diminished germinal centers and IgG production. This rules out a developmental defect in CRH-tg mice and demonstrates that elevated corticosterone is sufficient to impair this process.

The most interesting question is *how* do glucocorticoids impair germinal center formation? I have begun to address this question on a cellular basis by looking at FDC networks in corticosterone-treated mice. I found that, following immunization, FDC networks in corticosterone-treated mice are diminished based on histological markers and the ability to trap antigen-antibody complexes. This indicates that corticosterone decreases the number and/or activation state of FDC. Such an alteration in FDC may actually *cause* poor germinal formation or simply occur as a *result* of this impairment. However, preliminary evidence suggests that impaired FDC are causally related to the

germinal center defect because even in naïve mice, corticosterone treatment inhibited FDC function. Thus, FDC networks are altered basally, independent of immune activation.

Several possible mechanisms could explain the effects of glucocorticoids on FDC. As described in chapter 3, NF-κB expression by stromal cells is necessary to maintain FDC in a differentiated, functional state. Thus, glucocorticoids may directly affect FDC via inhibition of NF-κB signaling. In addition, glucocorticoids may suppress B cell factors required to maintain and activate FDC. These factors include lymphotoxin-α and TNF, which are known to be negatively regulated by glucocorticoids (311, 365). Finally, the apparent decrease in FDC markers and functional capacity could reflect a decrease in FDC numbers rather than a decreased maturation or functional state on a per cell basis.

The altered FDC network seen in naïve corticosterone-treated mice suggests that impaired FDC contribute to the germinal center defect. However, other factors may also be involved such that corticosterone would impair germinal center formation even if FDC networks were restored. I have not yet investigated other such influences, but inhibition of T cell help and glucocorticoid-mediated apoptosis of germinal center lymphocytes top the list of candidates, as described below.

Glucocorticoids and stress are associated with decreased T cell function, particularly suppression of CTL responses and Th1 type cytokines (see introduction). In addition, impaired T cell help has been implicated in effects of glucocorticoids on humoral immunity based primarily on the fact that TD responses are impaired much more severely than are TI responses. Similarly, diminished T cell help for newly activated B cells could impair the ability of those B cells to form germinal centers. Moreover, T cells are also important within germinal centers to provide help to the highest affinity

B cells that have "won" the competition to process and present antigen. Glucocorticoids may diminish expression of costimulatory molecules or cytokine production by helper T cells, limiting the ability of B cells to become activated to form germinal centers or preventing their survival once there.

In addition to functional effects, glucocorticoids may affect both T and B lymphocyte survival directly. Although mature peripheral lymphocytes are fairly resistant to glucocorticoid-induced apoptosis, germinal center lymphocytes are sensitive to these effects. In vivo treatment with glucocorticoids after germinal centers have developed causes degradation of germinal centers with morphological evidence of lymphocyte apoptosis (135, 136). More recently, Zheng showed that glucocorticoids given at the peak of the germinal center response (12 days after immunization) augment apoptosis of T cells within germinal centers (366). In addition, glucocorticoids antagonize the ability of BCR ligation to rescue isolated germinal center B cells from apoptosis in vitro (367). These studies used single high doses of synthetic glucocorticoids given during the germinal center response, while I used the endogenous glucocorticoid, corticosterone, given chronically starting well before immunization. Moreover, the same dose of corticosterone given at the time of immunization but not prior to caused only a subtle alteration in germinal center formation—germinal center numbers were unchanged and germinal center size was decreased only two-fold (see chapter three). Therefore, it is unlikely that the chronic corticosterone treatment in my experiments suppressed germinal centers primarily via apoptosis of lymphocytes already forming germinal centers. Rather, it appears that corticosterone may affect the lymphoid environment, rendering it non-conducive for germinal center formation upon immunization.

This can be tested in the future by treating mice with corticosterone prior to, but not following, immunization. If this has a more severe impact on germinal center formation than treatment after immunization, this would suggest that the prominent effect of corticosterone on germinal centers actually occurs prior to immune stimulation, rather than by affecting antigen-stimulated lymphocytes. The relative roles of microenvironment versus direct effects on lymphocytes can also be tested using fetal liver chimeras in which non-hematopoeitic cells are sensitive to glucocorticoid-induced immunomodulation, but hematopoeitic cells lack a functional glucocorticoid receptor (368). Because glucocorticoids play integral roles in shaping immune development and regulating inflammatory responses, I suspect that corticosterone exerts multi-faceted effects whereby it impairs the normal microenvironment necessary for germinal center formation and directly prevents full activation of lymphocytes following immunization.

Again, I propose a teleological argument for the sensitivity of germinal centers to glucocorticoids. This argument rests on the similarity between developing lymphocytes and germinal center B cells. In both cases, new antigen receptors are produced by a random process, and only those with an appropriate specificity become part of the repertoire. Those that are defective (unproductive rearrangements during lymphopoeisis and lost antigen specificity in the case of germinal center B cells) are deleted, as are those with self-specificity. Like positive selection in the thymus, endogenous glucocorticoids may be important during somatic hypermutation and affinity maturation to achieve the appropriate balance between survival of B cells with high affinity for antigen and deletion of B cells whose mutations produce self-reactive antibodies.

Neuroendocrine feedback during immune activation

The neuroendocrine and immune systems intimately regulate one another. Stress, including immune stress, such as infection or inflammation, stimulates the HPA axis. In turn, glucocorticoids released as the end product of HPA activation modulate many immune parameters, providing critical negative feedback to protect an organism against immune overshoot. Glucocorticoids down-regulate inflammatory mediators such as TNF and IL-1, thereby preventing cytokine-induced pathology and death. In addition to effects on these early inflammatory mediators, glucocorticoids also inhibit the Th1 cytokines, IFNγ and IL-12, which are toxic at high levels. Moreover, this results in down-modulation of the Th1 response, which may be important in preventing inflammatory-stimulated activation of autoreactive T cells that have escaped central deletion. For instance, in animal models in which endogenous glucocorticoid activity is limited, immunization with non-self antigens, such as streptococcal cell wall polysaccharide and CFA, can cause autoimmunity (46, 47, 95, 140). Thus, endogenous glucocorticoids released in response to immune stimulation are critical in limiting pro-inflammatory and Th1 responses to prevent deleterious consequences of overactivation of these systems.

In contrast, the rise in endogenous glucocorticoids due to immune activation *facilitates* antibody responses (67). Teleologically, this makes sense because 1) humoral responses are not characterized by dangerous levels of pro-inflammatory cytokines and 2) inadvertant activation of autoreactive B cells may be less dangerous in the absence of autoreactive T cells to provide help. Thus, activation of the HPA by immune stress provides a survival advantage via complex immune

regulation that is immunosuppressive or immunoenhancing depending on the balance between potential benefit and potential harm of a particular immune function.

Effects of non-immune stress on immunity

What is the immunological effect of HPA activation in response to *non-immune* type stress? It has been suggested that times of stress are associated with increased risk of infection. For instance, during both predator stress and social stress, wounding and subsequent infection is common. Similarly, during drought or other environmental stresses risks of parasitic or bacterial infection may be increased. Thus, general immunosuppression during stress does not seem advantageous. In fact, a common theme emerges that during stress innate immunity may be augmented while adaptive immunity is suppressed (see Figure 5, p. 116).

It may be advantageous to augment the first line of defense, which can mobilize quickly to contain infection before systemic spread. In addition, innate immunity has broad specificity to combat all classes of pathogens. HPA activation augments innate immunity in a variety of ways, most notably by increasing neutrophils and enhancing phagocytic capacity of neutrophils and macrophages (115, 116, 280-286). Certain subsets of lymphocytes also may be categorized as innate effectors—B-1 and marginal zone B cells, as well as intraepithelial and/or γδT cells. These cells bear antigen receptors that tend to recognize conserved pathogen moieties, such as phosphocholine, which is found on Gram positive and Gram negative bacteria, Trypanosomes, and nematodes (245). Thus, these classes of lymphocytes are protective against a variety of potential pathogens. Moreover, they are activated more quickly than classical lymphocytes, and they reside in sentinel locations such as the skin, gut, and marginal zone of the spleen.

Preliminary evidence supports the hypothesis that HPA activation may spare or even augment these populations of "innate" lymphocytes. I found that B-1 and marginal zone populations in CRH-tg mice are much less affected than are B-2 populations (unpublished observation). In addition, total circulating antibody prior to immunization is actually increased in CRH-tg mice (chapter 2). In a pathogen-free environment, such natural antibodies are thought to arise primarily from B-1 and marginal zone B cells (369). Moreover, the response to a prototypic B-1 antigen, PC, coupled to KLH, is relatively normal in CRH-tg mice. In contrast, production of group II antibodies, which do not recognize PC and are thought to arise from B-2 populations, is poor in CRH-tg mice. In addition, others have shown that cellular responses in the skin are augmented by acute stress (120, 121), and small intestine intraepithelial lymphocyte subsets are highly resistant to glucocorticoid-induced cell death (370). Thus, immune effectors that provide first line defense, have broad specificity, and are activated quickly may be particularly important to host defense during stress.

In contrast, "classical" adaptive immunity generally is decreased during stress. As described above, glucocorticoid-mediated regulation of certain aspects of adaptive immunity during immune activation provides a survival advantage by limiting inflammation and preventing autoimmunity. However, these preparative features may be optimized for short-term stress, and chronic HPA activation preceding immune challenge may suppress adaptive immunity to a deleterious degree. This has been shown convincingly for cytotoxic responses wherein stress-induced suppression of CTL generation correlates with increased pathogenesis and viral replication in HSV and flu infections (102, 327, 328). Likewise, we have shown that chronic HPA activation suppresses humoral immunity via inhibition of germinal center formation, which in turn leads to poor isotype switching and memory.

However, environmental stress did not affect germinal center formation despite high circulating levels of corticosterone. If HPA activation suppresses both cellular and humoral immunity, why does stress have a more profound effect on the former? As described earlier, overactivation of cellular immunity may be more dangerous than overactivation of humoral immunity. Moreover, one could argue that bacterial, rather than viral, infections are more likely to accompany many stresses such as predation, social battles, and other physical stresses that cause wounding. Antibodies, rather than CTL, are more critical to defense against common environmental bacteria, most of which are extracellular. Thus, overactivation of cellular immunity carries a high risk of autoimmunity, while inhibition of cellular immunity during stress may be only moderately detrimental. In contrast, overactivation of humoral immunity may carry a lower risk of autoimmunity, while inhibition of humoral immunity during stress may severely compromise host defense in the face of infection. In short, glucocorticoid-induced suppression of humoral immunity carries a high risk to benefit ratio. In response to this pressure, evolution may have engineered a mechanism whereby other stress-responsive pathways allow humoral responses to partially circumvent the suppressive effects of glucocorticoids.

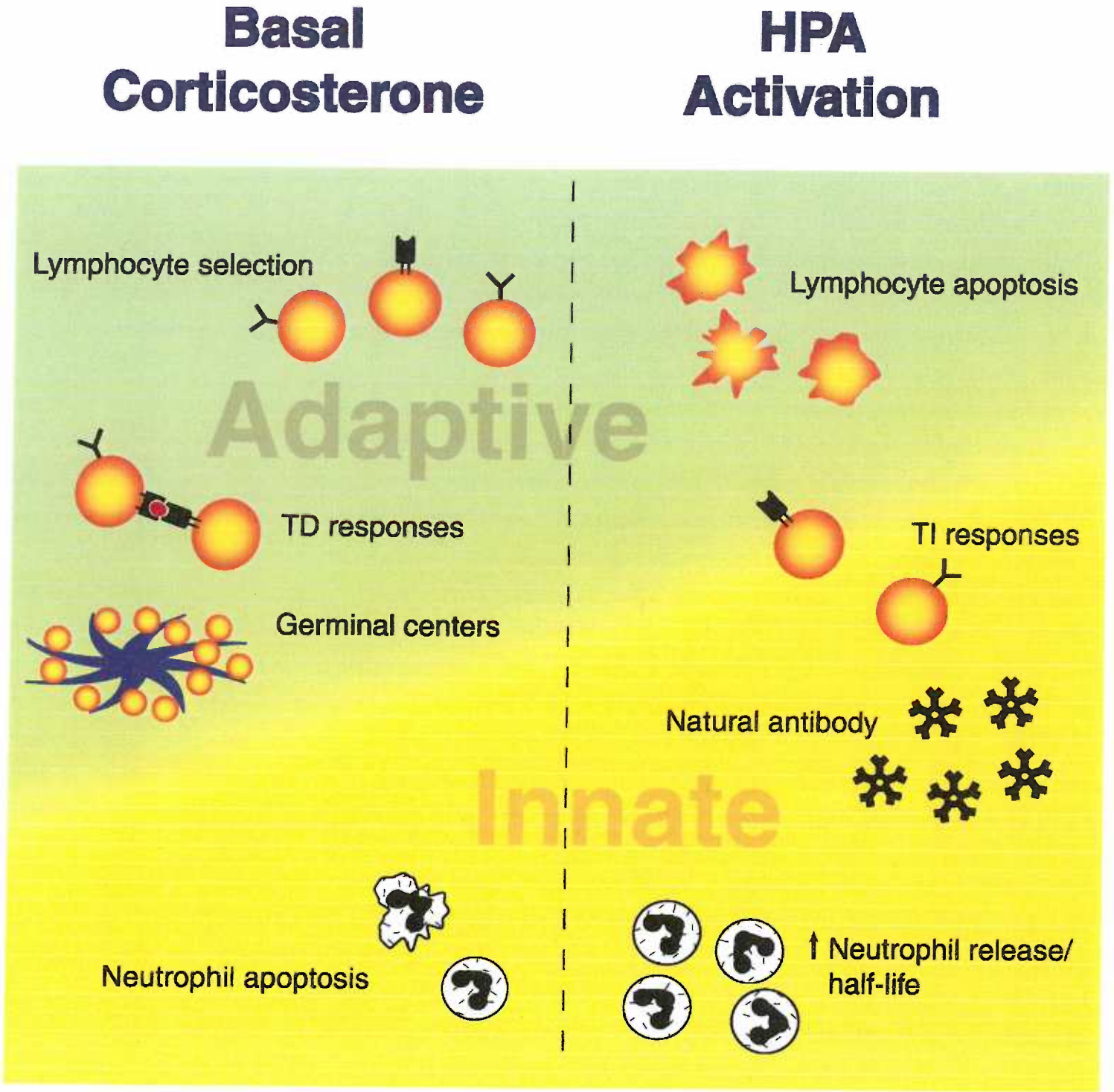

Figure 5. Schematic representation of effects of basal versus elevated levels of corticosterone on immunity. Basal levels of corticosterone (left) enhance development and responsiveness of adaptive immunity, while elevated levels of corticosterone (right) suppress adaptive immunity but enhance innate immunity. Thus, the balance between adaptive (green) and innate (yellow) immunity varies depending on the state of HPA activation.

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