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Review article

Local glucocorticoid production in lymphoid organs of mice and birds: Functions in lymphocyte development

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ABSTRACT

Circulating glucocorticoids (GCs) are powerful regulators of immunity. Stress-induced GC secretion by the adrenal glands initially enhances and later suppresses the immune response. GC targets include lymphocytes of the adaptive immune system, which are well known for their sensitivity to GCs. Less appreciated, however, is that GCs are locally produced in lymphoid organs, such as the thymus, where GCs play a critical role in selection of the T cell antigen receptor (TCR) repertoire. Here, we review the roles of systemic and locally-produced GCs in T lymphocyte development, which has been studied primarily in laboratory mice. By antagonizing TCR signaling in developing T cells, thymus-derived GCs promote selection of T cells with stronger TCR signaling. This results in increased T cell-mediated immune responses to a range of antigens. We then compare local and systemic GC patterns in mice to those in several bird species. Taken together, these studies suggest that a combination of adrenal and lymphoid GC production might function to adaptively regulate lymphocyte development and selection, and thus antigen-specific immune reactivity, to optimize survival under different environmental conditions. Future studies should examine how lymphoid GC patterns vary across other vertebrates, how GCs function in B lymphocyte development in the bone marrow, spleen, and the avian bursa of Fabricius, and whether GCs adaptively program immunity in free-living animals.

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Contents

1. Introduction

Glucocorticoids (GCs) are steroid hormones that are synthesized by the adrenal glands and released into the systemic circulation to regulate organismal physiology. Like all steroids, GCs are made from cholesterol, *via* a series of enzymatic conversions (Fig. 1). In some species, such as reptiles, amphibians, birds, and laboratory mice and rats, the major adrenal-derived GC in the blood is corticosterone, while in other species, such as fish, hamsters and primates, the major adrenal-derived GC is cortisol. Both corticosterone and cortisol bind the GC receptor (GR) and the mineralocorticoid receptor (MR). Upon ligand binding of the GR or MR, these receptors enter the nucleus and regulate transcription of a variety of genes affecting metabolism, inflammation, and other physiological processes (Bledsoe et al., 2002; Gomez-Sanchez and

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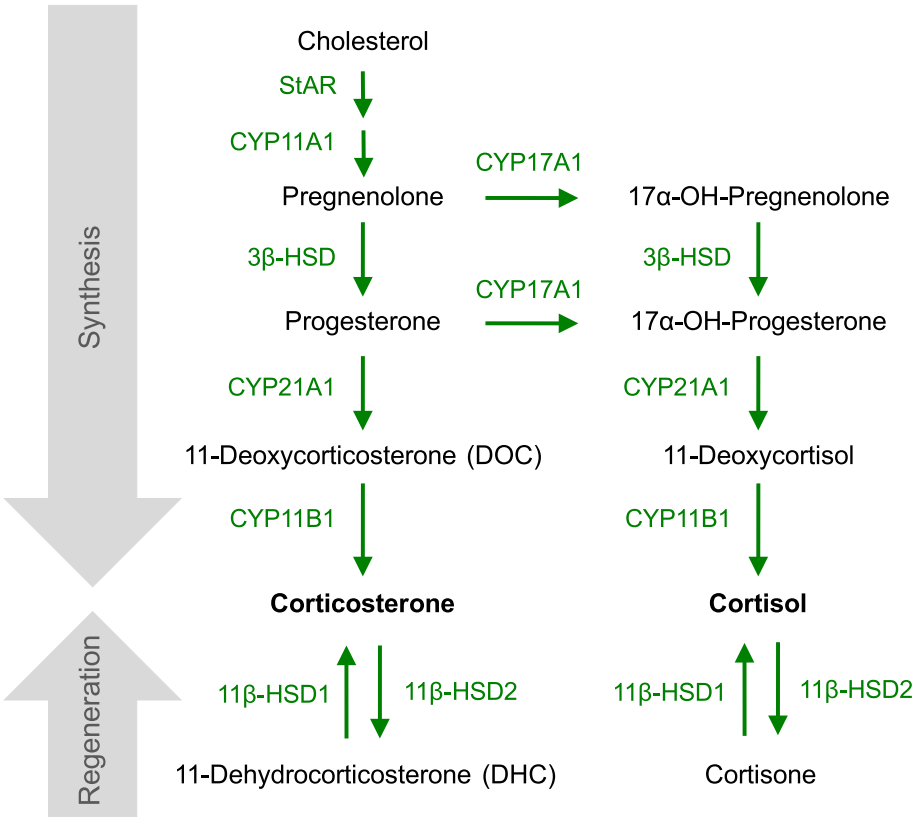

Fig. 1. Simplified glucocorticoid-metabolic pathway. Steroid names are in black, with the major active glucocorticoids bolded, and steroid-metabolic enzymes and their corresponding activities are in green. We define upstream precursor conversion into active glucocorticoids as "synthesis" and downstream metabolite conversion into active glucocorticoids as "regeneration." The combined contributions of synthesis and regeneration are together referred to as glucocorticoid "production." Adapted with permission from Taves et al., 2016b.

Gomez-Sanchez, 2011; Grossmann et al., 2012). Glucocorticoids can also signal *via* membrane-associated GRs (Vernocchi et al., 2013). GC secretion by the adrenal glands is strongly stimulated by various stressors, and GCs are best known for their role as mediators of the stress response. GCs classically function as systemic signals, acting on virtually all cells of the body to coordinate the organismal response to stressors. Among the many actions of GCs, one of the strongest is suppression of the immune system. First recognized by Selye (1936), an early bioassay for GC production was involution of immune tissues. Identification of adrenal GCs as the drivers of thymic involution (Ingle, 1940; Wells and Kendall, 1940) was followed by use for treatment of rheumatoid arthritis, for which they were remarkably effective (Hench et al., 1949). GC effects in different diseases and immune cells have been extensively studied since this initial work, and GCs are recognized as having potent suppressive activity across a range of immune cell subsets.

The immunosuppressive actions of GCs are critical in regulating the course of the immune response. Adrenal GC secretion is strongly induced by immune responses to a wide range of stimuli (*e.g.*, Besedovsky et al., 1975; Smith et al., 1982; Miller et al., 1997; Jamieson et al., 2010). In various models of infection and disease, reduced GC signaling (*e.g.*, *via* adrenalectomy, inhibition or genetic deletion of the GR) results in excessive secretion of pro-inflammatory cytokines, uncontrolled inflammation, and mortality (*e.g.* influenza, Avitsur et al., 2006; Jamieson et al., 2010; toxoplasmosis, Kugler et al., 2013; sepsis, Brewer et al., 2003; Li et al., 2015; trypanosome, Roggero et al., 2006). Thus, a critical role of GCs is to prevent over-shoot of the immune response (Sapolsky et al., 2000). However, there is a growing appreciation that GCs also have actions that promote effective immunity. Adrenal GCs promote the mobilization and redistribution of various immune cell subsets (Dhabhar et al., 1996; Bilbo and Nelson, 2003) and may enhance lymphocyte proliferation in the early stages of an immune response (Wiegers et al., 1994). GC upregulation of certain receptors, such as the interleukin 7 receptor, on effector lymphocytes might even serve to prime lymphocytes for more effective generation of immune memory (Franchimont et al., 2002; Lee et al., 2005). Thus, moderate and short-term elevations of circulating GCs can promote immunity, while high and longer-term elevations of circulating GCs suppress immunity (Martin, 2009).

Laboratory studies of conventional animal models, along with comparative studies of domestic and wild species, are complementary and important for understanding environmental regulation of immune development and immune responses. In this review, we will begin by focusing on data from laboratory mice, where GC actions in immunity are best understood, and then transition to discussing data from a range of bird species. We then outline some compelling areas for future investigation and how work in a variety of species and contexts can begin to address these important gaps in our knowledge.

2. Glucocorticoids and lymphocyte development in mice

GCs, in addition to regulating immune responses, are also well known for having potent effects on developing immune cells, especially lymphocytes. This may be best exemplified by the dramatic stress-induced involution of the thymus, which is the development site of T lymphocytes (T cells). T cells, along with B cells, are the effector cells of the adaptive immune system. While an ever-growing number of functions are attributed to T cells (Raphael et al., 2015), the two major conventional T cell subsets function in cell-mediated immunity (such as killing of pathogen-infected cells; $CD8^+$ cytotoxic T cells) and in secreting a variety of cytokines to determine the overall type of immune response ($CD4^+$ helper T cells).

Across vertebrates, the thymus is dedicated to the development and selection of T cells, which are derived from bone marrow precursors (Palmer, 2013). Developing T cells (thymocytes) use random

recombination of gene segments to generate an immensely diverse repertoire of T cells with unique T cell antigen receptors (TCRs). This random recombination allows an organism to theoretically generate 10^{15} different TCR specificities (Davis and Bjorkman, 1988) and a TCR repertoire that is capable of recognizing peptide fragments (presented by major histocompatibility complex molecules; MHCs) from any pathogen it might encounter. This process of random recombination, however, results in many non-functional TCRs and some autoreactive TCRs, which must both be removed. This removal occurs during the process of thymocyte selection, where the TCRs of developing thymocytes are presented a range of self-antigens by MHC molecules in the thymus (Klein et al., 2009). The majority of thymocytes that receive minimal or no TCR signals die; thymocytes that receive moderate TCR signals are positively selected and survive; and thymocytes that receive strong TCR signals are negatively selected and die (Daniels et al., 2006) or diverge to alternate fates, such as the regulatory T cell (Treg) lineage (Malchow et al., 2016). Proportions of unselected, positively selected, and negatively selected TCRs have been estimated at 85%, 7.5%, and 7.5%, respectively (McDonald et al., 2015).

Beginning in the early 1990s, it was discovered that TCR and GR signals, which individually induced thymocyte apoptosis, together resulted in thymocyte survival (Iwata et al., 1991). This mutual antagonism followed a dose-dependent pattern, with higher GC concentrations protecting from higher strength TCR stimuli (Iwata et al., 1991). This led to the surprising discovery that metyrapone inhibition of GC production *in vitro*, and transgenic reduction of GR expression *in vivo*, actually increased apoptosis in thymocytes undergoing selection ($CD4^+CD8^+$ double positive thymocytes) (Vacchio et al., 1994; King et al., 1995). Reduced GR expression also dramatically reduced thymus size due to a loss of double positive thymocytes (King et al., 1995).

More detailed analyses found that reduced GC levels (Vacchio et al., 1994, 1999; Vacchio and Ashwell, 1997) or GR expression (Lu et al., 2000) both resulted in negative selection of specific TCRs that would otherwise be positively selected (Fig. 2). Together, these data indicated that GC antagonism of TCR signaling results in positive selection (and inclusion in the mature repertoire) of TCRs that would otherwise be eliminated by negative selection. Thymic GCs are thus required to generate a T cell repertoire with effective TCR signaling in response to antigen:MHC (reactivity), and resulting T cell activation, proliferation, and effector differentiation (responsiveness) to certain antigens and pathogens (Mittelstadt et al., 2012; Fig. 3). Reduced GR expression also ameliorated pathology in a model of autoimmunity (Tolosa et al., 1998).

Parallel to T cell development in the thymus, mammalian B cell development occurs in the bone marrow (also the origin of other hematopoietic cell lineages). Antibodies directly recognize target epitopes (rather than recognizing peptide fragments presented by MHC) and therefore B cell selection does not need to ensure recognition of MHC, as with developing T cells. However, B cells still undergo positive and negative selection in the bone marrow (Cyster et al., 1996; Wardemann et al., 2003). Autoreactive B cell antigen receptors (BCRs; antibody molecules expressed on the B cell surface) can undergo receptor editing, which allows alteration of an autoreactive BCR into a weaker signaling BCR that can then be exported from the bone marrow (Gay et al., 1993; Tiegs et al., 1993). B cell maturation and selection continues in the spleen, where immature B cells become mature naïve B cells (Allman et al., 2001). Further editing of the BCR (and removal of newly-arising autoreactive B cells) occurs in the lymph nodes during an immune response (Ray et al., 1996; Hande et al., 1998).

As with developing T cells, GCs have potent pro-apoptotic effects on developing B cells. Chronic elevation of circulating GCs results in dramatic reductions in the number of bone marrow B cells, especially developing B cells (Garvy et al., 1993; Trottier et al., 2008). Developing B cells express GR and are highly sensitive to GC-induced apoptosis (Gruver-Yates et al., 2014; Taves et al., 2016b). In contrast to T cells, little is known about how GCs interact with BCR signaling in developing B cells. However, there are some indications that GCs might play a role in B cell development and selection. First, GCs can inhibit BCR signaling in leukemia cells (Cortez et al., 1996), suggesting that this is also possible in normal developing B cells. Second, the avian bursa of Fabricius (hereafter bursa), which functions solely in B cell development, is highly responsive to GCs (see below). Thus, GCs are likely to function, in ways that remain unclear, in B cell development.

3. Local *versus* systemic glucocorticoid regulation in mice

Across species, thymus size and production of T cells is greatest in early life, and thymus involution begins in adolescence and is driven by increased gonadal production of sex steroids (Chen et al., 2010; Palmer, 2013). Thymus involution also corresponds with adrenal GC production; thymus size is high in neonatal life, when circulating GCs are low and minimally responsive to stressors (termed the stress hyporesponsive period (SHRP); Meaney et al., 1985). Involution continues over the course of adult life. The thymus fills with adipose tissue, the number of exported T cells decreases, and new T cells are less able to respond effectively to infection (Calder et al., 2011; Palmer, 2013). These steady changes are associated with increases in basal circulating GCs with age (Meaney et al., 1992; Lupien et al., 1994; Taves et al., 2015). GC induction of thymic involution has led to the conclusion that GCs negatively affect T cell development. Furthermore, the near absence of

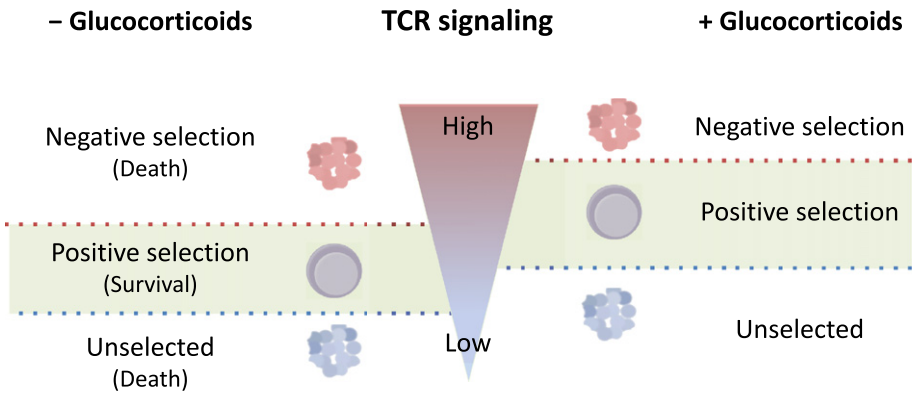

Fig. 2. Glucocorticoids antagonize T cell antigen receptor (TCR) signaling in the thymus to shift selection thresholds for survival and maturation of T cells. In the thymus, developing T cells (thymocytes) undergo strict selection to ensure functional TCR recognition of MHC molecules (positive selection), but to avoid strong activation in response to self-antigen:MHC (negative selection). Glucocorticoids antagonize TCR signaling, shifting the selection threshold to result in positive selection of some thymocytes with TCRs that would otherwise be negatively selected. Glucocorticoids may similarly result in death of unselected thymocytes with TCRs that would otherwise be positively selected, but whether this threshold shifts in parallel with that for negative selection is unclear. Adapted from Van Laethem et al. (2001).

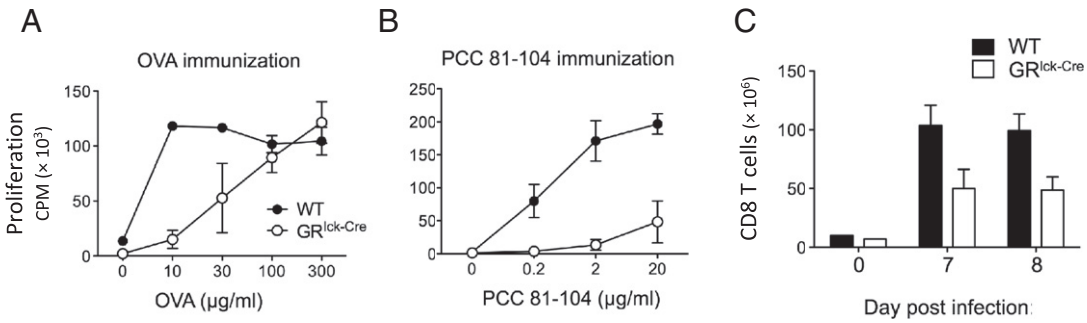

Fig. 3. Glucocorticoid signaling is critical for development of immunocompetent T cells. The requirement for glucocorticoid receptor (GR) signaling in thymocyte development has been demonstrated with multiple transgenic mouse models, most recently in mice with T cell-specific deletion of the glucocorticoid receptor ($GR^{lck\text{-}Cre}$). A, B) Wild-type (WT) or $GR^{lck\text{-}Cre}$ mice were immunized with one of two model antigens in Complete Freund's Adjuvant, Ovalbumin (OVA) or Pigeon Cytochrome C peptide fragment (PCC 81-104). 8–9 days later, T cells were harvested and incubated with antigen-presenting cells, the indicated dose of peptide, and 3H-thymidine to quantify proliferation (CPM = counts per minute). T cell proliferation was dramatically reduced in $GR^{lck\text{-}Cre}$ T cells, showing that the presence of the GR enhances T cell reactivity to these antigens. GR deficiency had no effect in mice with a fixed TCR repertoire (data not shown), demonstrating that glucocorticoid enhancement of T cell reactivity requires a diverse TCR repertoire. C) Lymph node $CD8^+$ (cytotoxic) T cells were collected after lymphocytic choriomeningitis virus (LCMV) infection, showing that the presence of the GR in T cells is responsible for an enhanced, rather than reduced, immune response to LCMV *in vivo*.
Reprinted with permission from Mittelstadt et al. (2012).

circulating GCs in early life (Wada, 2008) appears to be inconsistent with a critical role for GCs in T cell selection, as GCs would be especially important during this period of high T cell production.

In addition to acting as passive recipients of circulating steroids, tissues can also actively regulate their local steroid levels. Local steroid production could occur *via de novo* GC synthesis from cholesterol or *via* GC synthesis from circulating precursors (Davies and MacKenzie, 2003; Schmidt et al., 2008; Taves et al., 2011) (Fig. 4). Here, we use GC "synthesis" to refer to conversion of upstream precursors to GCs. Local steroid synthesis (extra-adrenal, extra-gonadal synthesis) was first suggested by the finding that dehydroepiandrosterone (DHEA), an androgen precursor, is locally elevated in the rat brain, compared to plasma

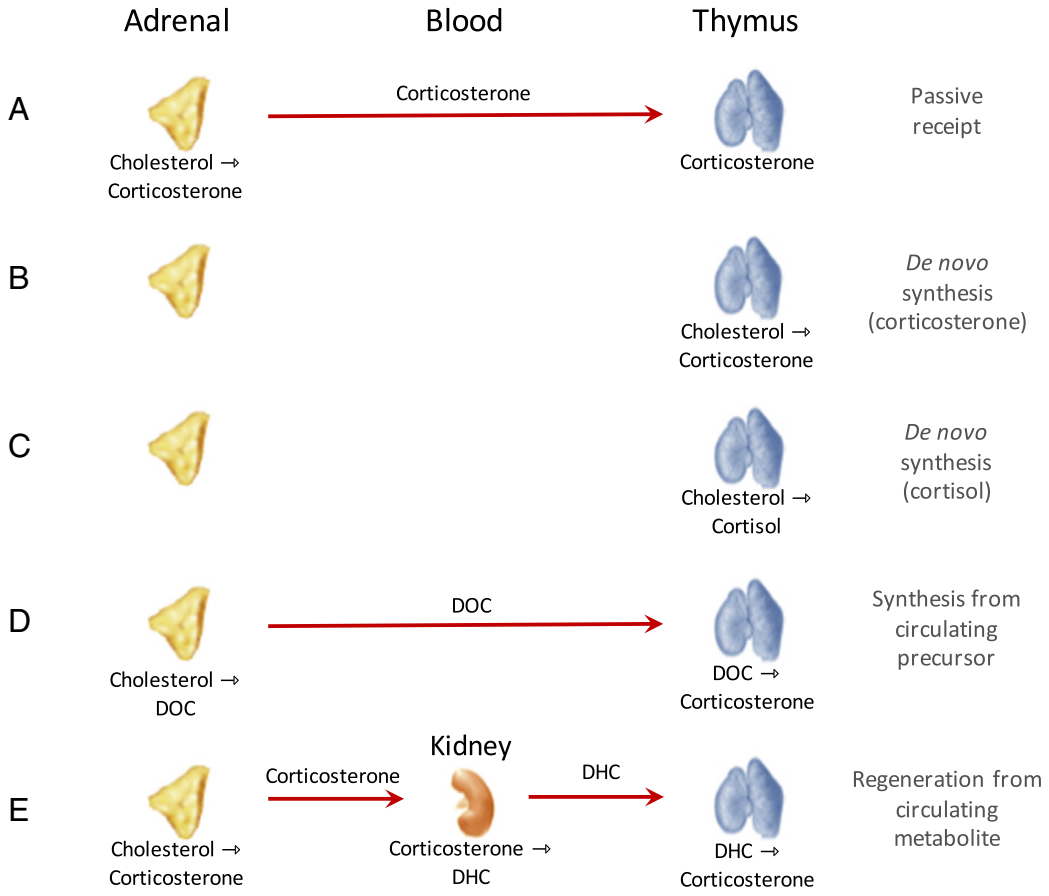

Fig. 4. Different models of glucocorticoid production and action in the thymus (and other lymphoid organs). A) Corticosterone, the major circulating glucocorticoid in mice and birds, is synthesized *de novo* from cholesterol in the adrenal glands, and travels through the blood to bind glucocorticoid receptors (GR) in the thymus. B) Corticosterone is synthesized *de novo* in the thymus. C) Corticosterone is synthesized in the adrenals, while an alternate glucocorticoid, cortisol, is synthesized *de novo* in the thymus. D) Glucocorticoid precursors, such as progesterone or DOC (11-deoxycorticosterone) are synthesized by the adrenal glands, and travel through the blood to the thymus, where they are converted to corticosterone. E) Corticosterone is synthesized by the adrenal glands, converted to inactive DHC (11-dehydrocorticosterone) by the kidneys and other tissues, and regenerated to active corticosterone in the thymus.

(Corpechot et al., 1981). It was later shown that steroids could be locally synthesized in the brain ("neurosteroids") (Le Goascogne et al., 1987; Hu et al., 1987). Local steroid synthesis was then demonstrated in the developing mouse thymus (Vacchio et al., 1994). Specifically, this work showed that cultured thymic epithelial cells converted a cholesterol analog to steroids, including DOC, and that this ability to synthesize steroids ("immunosteroids") decreased with age (Vacchio et al., 1994). Furthermore, locally-produced GCs protected cultured thymocytes against TCR-induced death *in vitro* (Vacchio et al., 1994). *In vitro* GC production by the mouse thymus was confirmed by two other laboratories (Pazirandeh et al., 1999; Lechner et al., 2000), one of which subsequently reported GC synthesis by thymocytes as well (Qiao et al., 2008). GC production by thymocytes, in contrast to that of thymic epithelial cells, increased with age (Qiao et al., 2008), and in males was stimulated by gonadal testosterone (Chen et al., 2010). All these studies used metyrapone as an inhibitor of CYP11B1, to block glucocorticoid synthesis, although metyrapone also inhibits 11β-HSD1 (Sampath-Kumar et al., 1997) (Fig. 1). GC synthesis was also shown in cultured chicken lymphoid organs, but this study suggested local production of cortisol rather than corticosterone (see below; Lechner et al., 2001).

To test whether local GC synthesis occurs *in vivo*, we collected thymus and circulating blood from mice throughout development (Taves et al., 2015). We also collected bone marrow and spleen, to see if these lymphoid organs might also produce GCs. We extracted steroids using solid phase extraction, separated steroids with high performance liquid chromatography (HPLC), and measured GCs and their upstream precursors with specific immunoassays. In early life, GC and GC precursor concentrations were higher in lymphoid organs than in circulating blood (Fig. 5, Taves et al., 2015). These data demonstrate that GCs are locally elevated *in vivo*, especially during development. Furthermore, while corticosterone, the major circulating GC in mice, was locally elevated in lymphoid organs, cortisol was even more dramatically elevated (Fig. 5, Taves et al., 2015). This is striking because cortisol is generally thought to be absent in the mouse (Touitou et al., 1990; van Weerden et al., 1992). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) verified the presence of cortisol in mouse lymphoid tissues

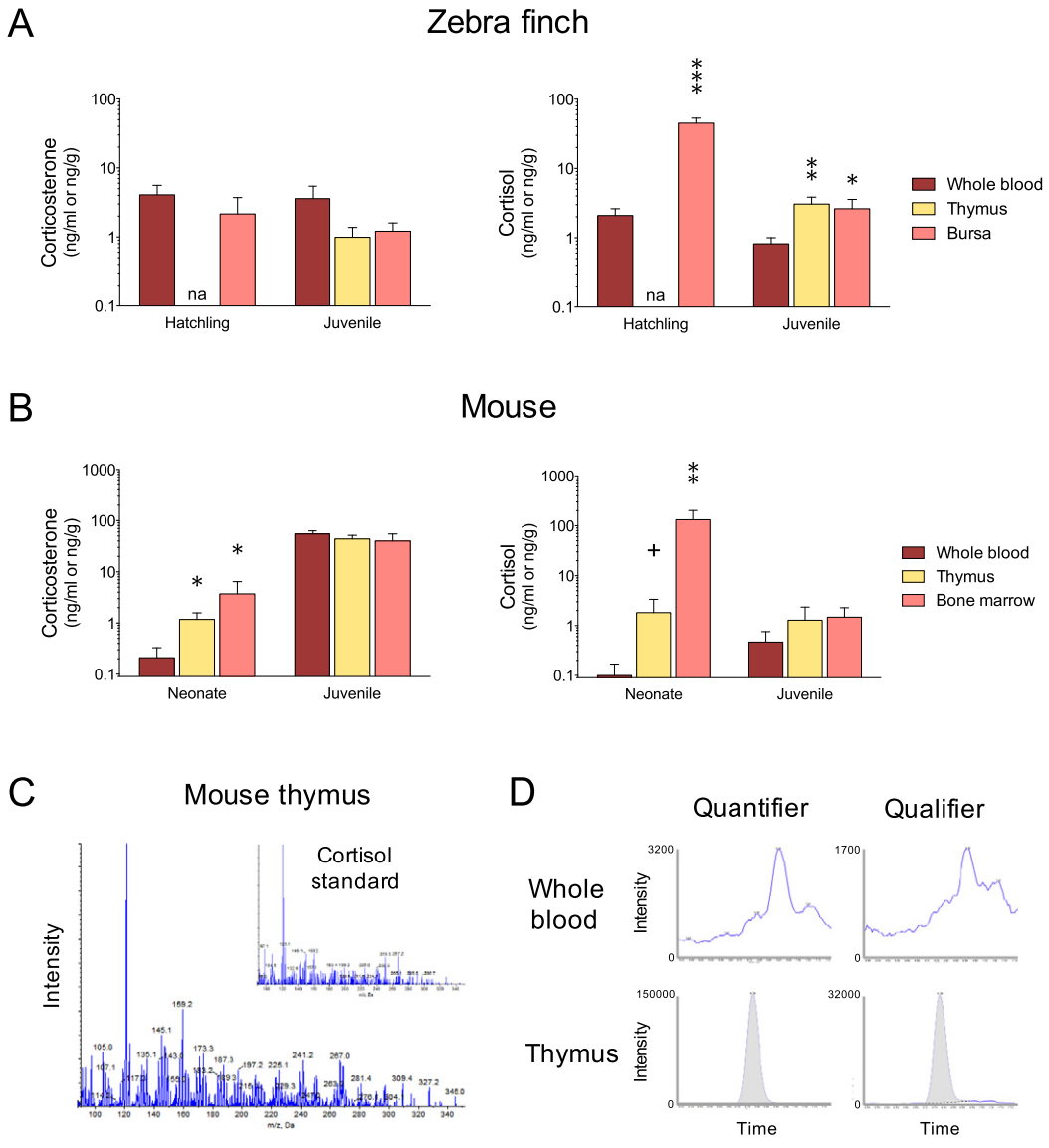

Fig. 5. Endogenous glucocorticoids are locally elevated in lymphoid organs of developing zebra finches and mice. A) Corticosterone and cortisol levels in whole blood, thymus, and bursa of Fabricius (bursa) of hatchling (day of hatch) and juvenile (posthatch day 30) zebra finches. na = not assessed. B) Corticosterone and cortisol levels in whole blood, thymus, and bone marrow of neonatal (postnatal day 5) and juvenile (postnatal day 15) C57BL/6 mice. Note the log scale on the y-axes of A and B. Steroids were isolated with solid-phase extraction followed by HPLC prior to immunoassay quantification. C) LC-MS/MS spectrograms and D) multiple reaction monitoring (MRM) detection of quantifier (m/z 363.4 → 121.1) and qualifier (m/z 363.4 → 97.1) transitions confirming the presence of cortisol in mouse thymus but not whole blood. Grey shaded regions represent cortisol product ions.
Data are adapted from Taves et al. (2015, 2016a), and zebra finch data closely match previous results from Schmidt and Soma (2008).

(Fig. 5, Taves et al., 2015). These data, together with detection of steroidogenic enzyme mRNA in lymphoid organs, suggested that local GC synthesis occurs *in vivo*, and that it can result in high endogenous concentrations of cortisol, as well as corticosterone.

As the thymus functions solely for T cell development, it is extremely likely that thymic GC production is involved in T cell development and/or selection. Thymic GC synthesis could be important in this process for several reasons. First, it could maintain locally-required GC levels even when circulating GCs are minimal, as during the SHRP in early development (Taves et al., 2011a). Even in the presence of circulating adrenal-derived GCs, it could function to maintain locally high concentrations, which might buffer the thymus from changes in adrenal GC production, therefore maintaining relatively consistent GC levels for T cell selection requirements. This is supported by the finding that adrenocorticotropic hormone (ACTH), which stimulates adrenal GC production, has little or no effect on thymus GC production (Vacchio et al., 1994; Pazirandeh et al., 1999), and also by the finding of GC production by the thymus of the chicken, which does not have a clear SHRP during early development (Freeman and Flack, 1980; Freeman and Manning, 1984). Finally, local GC synthesis can allow production of alternate steroids, such as cortisol in mice.

In addition to GC synthesis from precursors, GCs can also be locally produced *via* "regeneration," conversion of the inactive GC metabolites 11-dehydrocorticosterone (DHC) or cortisone into active GCs (corticosterone or cortisol, respectively) (Figs. 1, 4). Circulating GCs, primarily from the adrenals, are converted by 11β-HSD2 to inactive DHC or cortisone. This inactivation occurs predominantly in the kidney, colon, and placenta, but 11β-HSD2 is also expressed in other organs, including the ovary and brain (Roland and Funder, 1996; Wyrwoll et al., 2015). DHC and cortisone might also be produced by the adrenals (Shimojo et al., 1996). Unlike active GCs, which in plasma are largely bound to corticosteroid-binding globulin (CBG) in mammals, GC metabolites do not bind CBG with high affinity and can enter tissues more readily (Dunn et al., 1981). Within lymphoid organs, 11β-HSD1 can then convert DHC into active corticosterone (or cortisone into cortisol).

While we had found locally elevated GCs in murine bone marrow and spleen (Taves et al., 2015), it was not clear if this was, like in the thymus, due to local production or some other mechanism (*e.g.*, sequestration). We therefore aimed to test whether one of the pathways in Fig. 4 might underlie endogenous GC elevation in bone marrow. Additionally, previous work on GC synthesis in the thymus used metyrapone as a CYP11B1 inhibitor. However, in addition to inhibiting CYP11B1, metyrapone also inhibits GC regeneration *via* 11β-HSD1 (Sampath-Kumar et al., 1997). As 11β-HSD1 has been reported in the murine thymus (Zhang et al., 2005; Nuotio-Antar et al., 2006), we also compared the relative contributions of CYP11B1 and 11β-HSD1 to thymic GC production. In all three lymphoid organs examined, there was little gene expression or enzyme activity of CYP11B1, and much higher gene expression and enzyme activity of 11β-HSD1 (Taves et al., 2016b). In the presence of either exogenous precursor (DOC) or metabolite (DHC), thymus and bone marrow both preferentially produced corticosterone *via* regeneration. In general, expression of CYP11B1 decreased with age, while 11β-HSD1 increased with age, suggesting that synthesis might be more important in early development, while regeneration might be more important during maturation and adulthood (Taves et al., 2016b). Consistent with this, testosterone increases 11β-HSD1 gene expression in the thymus (Chen et al., 2010). Lymphoid 11β-HSD1 enzyme activity is physiologically relevant, as physiological concentrations of DHC resulted in 11β-HSD1- and glucocorticoid receptor-dependent T and B lymphocyte apoptosis *in vitro*, as quantified by flow cytometry detection of caspase activity and cell death (Taves et al., 2016b) (Fig. 6). The relative contributions of local GC synthesis and regeneration *in vivo*, however, must still be tested.

In the thymus, GC regeneration suggests very different physiological regulation than does GC synthesis. Instead of being "autonomous," thymic GC regeneration is instead dependent on circulating DHC or cortisone, which can change in response to environmental stressors (Harris et al., 2001; Obut et al., 2004, 2009). Thus, local GC regeneration, rather than minimizing variation in thymus GC concentrations, might actually amplify the effects of systemic GC changes on the thymus. This could partly explain why the thymus is so highly responsive to stressors. Such responsiveness also suggests an adaptive role for changing the TCR signaling threshold depending on an individual's environment, especially during early life. However, as few studies have

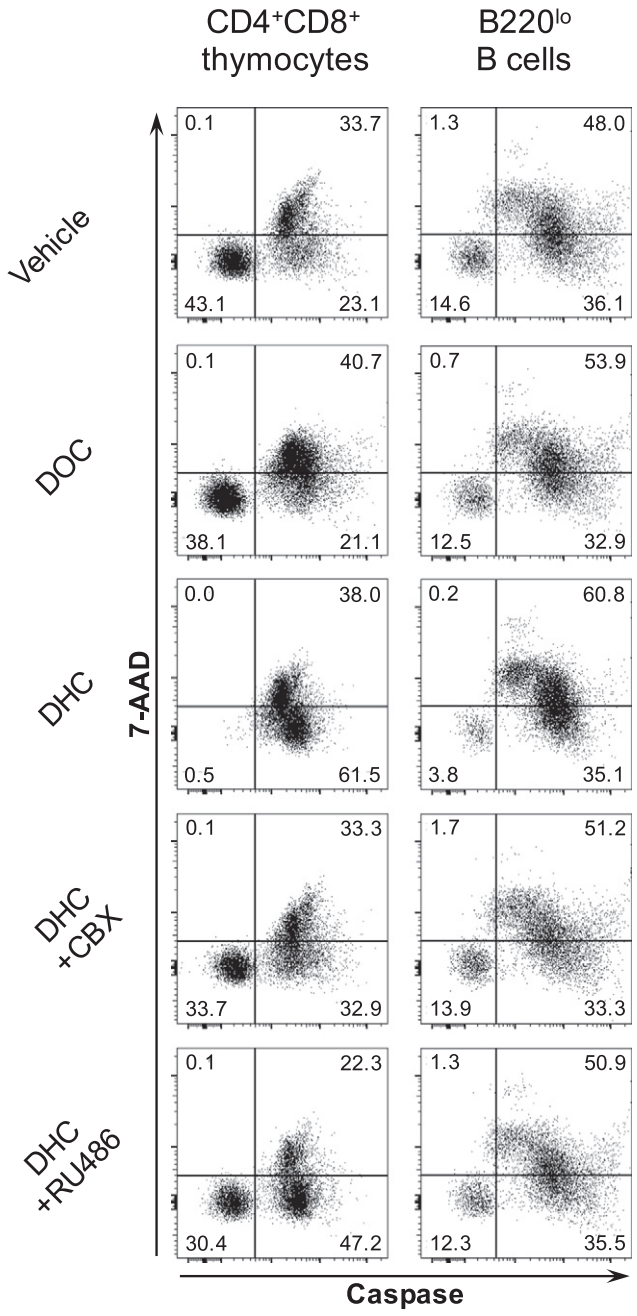

Fig. 6. Locally-regenerated glucocorticoids can induce apoptosis of developing lymphocytes. Thymus or bone marrow cells from adult mice were incubated for 24 h in 100 nM 11-deoxycorticosterone (DOC) or 11-dehydrocorticosterone (DHC), with 1 mM carbenoxolone (CBX, an 11β-HSD inhibitor) or 1 μM RU486 (a glucocorticoid receptor antagonist). Cells were stained with antibodies against CD4, CD8, and B220 (to identify developing lymphocytes), 7-AAD (to detect dead cells) and caspase 3/7 substrate (to detect induction of apoptosis), and measured by flow cytometry. Numbers indicate the percent of cells in the corresponding quadrant. DHC but not DOC potently induces apoptosis of developing T and B cells, and this is dependent on 11β-HSD1 activity and glucocorticoid receptor binding.
Data are adapted from Taves et al. (2016b).

measured circulating DHC in mice (Harris et al., 2001; Hundertmark et al., 2002; Tagawa et al., 2007), it is unclear how levels change over development, in response to various stimuli, and how they are regulated.

The function of local GC regeneration in the bone marrow might be similar to that of local regeneration in the thymus – that is, amplification, rather than dampening, of tissue sensitivity to environmental stressors and associated increases in circulating GCs and GC metabolites. Regulation of B cell development and selection is one potential function of local GCs, but the varied functions of bone marrow suggest that there may be other functions. Such other functions include stress-induced increases in erythropoiesis (Bauer et al., 1999). An increase in red blood cells can be highly adaptive under longer-lasting stressful conditions, so local GC regeneration could result in bone marrow being especially sensitive to GCs and thus adaptively increase erythrocyte production. Production of innate immune cells (Trottier et al., 2008) might also play a preparative role, whereas differentiation of osteoblasts to osteoclasts (Cooper et al., 2002) and corresponding inhibition of bone growth might contribute to organismal redirection of resources to more immediately critical functions.

4. Lymphoid glucocorticoids across species and environments

Lymphocyte development is best understood in laboratory mice, but lymphocyte development in other jawed vertebrates also involves random generation of antigen receptors and subsequent selection for appropriately reactive antigen receptors. The avian adaptive immune system, as in mammals, is characterized by T and B cell lineages, which derive from bone marrow precursors and develop in different compartments. In birds, T cell development and selection occurs in the multi-lobed thymus, while B cell development and selection occurs in the bursa (Glick et al., 1956; Ratcliffe, 2006). Chicken thymus and bursa exhibit GC-synthetic enzyme activities, producing GCs *in vitro* (Lechner et al., 2001), which suggests that GCs regulate both T and B cell development, potentially by affecting selection, as in the mouse thymus. Furthermore, while the chicken adrenals produce corticosterone, the chicken lymphoid organs produce cortisol *in vitro* (Lechner et al., 2001). GC production in the avian bursa is especially intriguing, as this organ is dedicated to B cell development, unlike mammalian bone marrow, and so gives stronger support to the hypothesis that GCs regulate B cell development. Avian lymphocytes are also sensitive to GC-induced apoptosis, with cortisol being more potent than corticosterone (Compton et al., 1990). Also, avian lymphoid organs undergo GC- and stress-induced involution (Puvadolpirod and Thaxton, 2000), further indicating that GC signaling is likely to play similar roles in avian and mammalian lymphocyte development.

To explore whether GCs are locally produced *in vivo* by avian lymphoid organs, we used solid phase extraction and specific immunoassays to investigate whether lymphoid organs (thymus, bursa, and spleen) of the zebra finch (*Taeniopygia guttata*) have elevated GC concentrations. The zebra finch is a songbird species that is commonly used in laboratory studies (Griffith and Buchanan, 2010). Developing finch lymphoid organs have dramatically elevated GC levels, especially at hatch (Schmidt and Soma, 2008; Taves et al., 2016a). Intriguingly, while the major circulating GC in finches is corticosterone, lymphoid organs of hatchling finches have higher concentrations of cortisol (Fig. 5A). These patterns are remarkably similar to those in neonatal mice (Fig. 5B) and are consistent with local GC production occurring in non-mammalian vertebrates. These parallels furthermore suggest that GCs play a conserved role in lymphocyte development and that cortisol could have a separate function from corticosterone in corticosterone-dominant species. This could occur if cortisol and corticosterone show differential binding to receptors in lymphoid organs. To explore this possibility, we examined corticosterone and cortisol binding in cell membranes and cytosol from juvenile finch lymphoid tissues. While both corticosterone and cortisol bind to GR-like sites in the cytosol, only corticosterone binds to MR-like sites in the cytosol, and only cortisol binds to GR-like sites in the bursa membranes (Table 1; Schmidt et al., 2010). Corticosterone and cortisol might therefore signal *via* distinct pathways, rather than being functionally equivalent.

We also compared endogenous lymphoid GCs in the developing zebra finch with those in the wild European starling (*Sturnus vulgaris*), which is also altricial (Schmidt et al., 2009). Unexpectedly, we found little evidence for locally elevated GCs, either cortisol or corticosterone, in lymphoid organs of hatchling or juvenile starlings. While corticosterone concentrations were usually higher in plasma than in lymphoid organs, cortisol concentrations were similar in both. Thus, there might be differential regulation of these GCs in starling lymphoid organs.

Both the zebra finch and mouse are altricial species, and locally elevated GC levels are most pronounced in early life, during the SHRP. To test whether local elevation of GCs also occurs in lymphoid organs of precocial species, which are not known to experience an SHRP, we used solid phase extraction, HPLC, and specific immunoassays to compare *in vivo* lymphoid GC levels of zebra finches to those of two precocial domestic avian species, the domestic chicken (*Gallus domesticus*) and the Japanese quail (*Coturnix japonica*). Two strains of chicken (White Leghorn and Rhode Island Red) and of quail (UBC and Hawaiian lines) were included, so within-species differences could be correlated with known differences in immunity. As before, cortisol was elevated in the thymus and bursa of developing finches. In contrast, in hatchling chicken and quail, lymphoid GC levels were similar to plasma GC levels, with little cortisol detected (Taves et al., 2016a). No GC differences were detected between strains of the same species. These data suggest that local GC production may play a lesser role in species whose adrenals are fully active in early development, such as those with a precocial pattern of development. These data also indicate that cortisol is not produced in lymphoid organs of all birds.

While there is evidence for lymphoid GC production in laboratory mice and zebra finches, lymphoid GC patterns *in vivo* are highly variable. We have begun to explore environmental factors that might regulate lymphoid GC levels. For instance, we have found that neonatal mice in one animal facility had locally elevated corticosterone and cortisol, while the same strain of mice in a different animal facility at the same institution had locally elevated corticosterone only (Taves, 2015; Taves et al., 2015; Taves et al., 2016b). One major difference between these animal facilities was their microbe exclusion lists (Taves, 2015). In a recent study, the microbiota from pet store mice profoundly altered the immune system of laboratory mice, including pathogen-specific immune responses (Beura et al., 2016). Furthermore, hatchling zebra finches from a colony infected with *Mycobacterium avium* had locally elevated corticosterone rather than locally elevated cortisol (Taves and Soma, unpublished data), while locally elevated cortisol was present in finch hatchlings from uninfected colonies in two different institutions (Schmidt and Soma, 2008; Taves et al., 2016a). Taken together, these data suggest that microbiota regulate lymphoid GC production and

Table 1
Corticosterone and cortisol binding sites in juvenile zebra finch plasma and lymphoid tissues.

Tissue compartment	Binding site	Affinity (K_d, nM)		Maximum binding (fmol/mg protein)	
		Corticosterone	Cortisol	Corticosterone	Cortisol
Plasma		1.4	1.3	**69.0**	**144.6**
Bursa cytosol	I	**0.5**	**nd**	**35.3**	**nd**
	II	26.3	12.8	386.5	242.9
Thymus cytosol	I	**0.1**	**nd**	**26.0**	**nd**
	II	11.6	9.2	**226.6**	**77.7**
Bursa membrane		**nd**	**5.4**	**nd**	**31.7**

Tissues were collected from zebra finches at posthatch day 30. Binding site indicates Type I (mineralocorticoid receptor)-like or Type II (glucocorticoid receptor)-like binding affinity, when two binding sites were predicted. No thymus membrane binding sites were detectable, possibly due to the very small tissue sizes. Bold values indicate a significant difference between corticosterone and cortisol, and nd indicates that the indicated binding site was nondetectable. Data are from Schmidt et al. (2010).

which GCs are produced. Work in both the laboratory and the field will be important to examine environmental regulation of lymphoid GCs. Laboratory studies offer the ability to strictly control and experimentally manipulate environmental variables, such as diet, pathogen exposure, and microbiome, while field studies offer the ability to compare closely-related species along a gradient of developmental life history patterns or test one species living in different conditions, such as small *versus* large breeding colonies.

5. Implications for integrative biologists

The finding that lymphoid GC levels can shift selection thresholds for developing T cells (and possibly B cells) presents a testable model in which chronically elevated lymphoid GC levels, especially during early life, are predicted to have long-lasting or "programming" effects by increasing the net reactivity of the lymphocyte repertoire (Fig. 7). Some studies examining developmental GC effects on immune development support the model in which increased GC exposure results in increased T cell reactivity. For example, while endogenous GCs prevent lethality of experimental autoimmune encephalomyelitis (EAE, a rodent model of multiple sclerosis) (Dowdell et al., 1999), neonatal GC treatment increases EAE susceptibility in adult rats (Bakker et al., 2000), and reduced GR expression decreases EAE susceptibility in mice (Marchetti et al., 2002). This could be due to greater T cell affinity for EAE autoantigen. Similarly, in humans, prenatal GC treatment increases the risk of autoimmune type I diabetes later in life (Greene et al., 2013). In contrast, prenatal GC treatment decreases lymphocyte reactivity to allogeneic leukocytes in rhesus monkeys (Coe et al., 1999), demonstrating reduced T cell recognition of MHC-presented antigen. These contrasting results could be a function of the timing of GC administration, as fetal T cells preferentially differentiate into regulatory T cells (Tregs) while postnatal T cells differentiate more into conventional T cells (Mold et al., 2010). Variability might also point to a dose-dependent change in the GC effect on lymphocyte selection, where moderate changes in GC levels shift the thymocyte selection threshold, but very high GC levels (which dramatically reduce thymus size; Avitsur et al., 2016) reduce T cell production and responsiveness. Such reduced immune reactivity might not be optimal, but rather a result of homeostatic overload (Romero et al., 2009). These models could be tested in different species by examining antigen receptor-dependent assays of lymphocyte function, such lymphocyte proliferation in response to allogeneic leukocytes, immunization with specific antigens, or infection with viral pathogens. Notably, such tests of antigen-specific T cell responses can be performed in a range of species, in the field and laboratory (Demas et al., 2011).

Birds are particularly well-suited for studying the roles of GCs in early-life immune programming, particularly in B cell selection. Studies of captive-bred and wild songbirds have been instrumental in understanding how GCs affect early development (Buchanan et al., 2004;

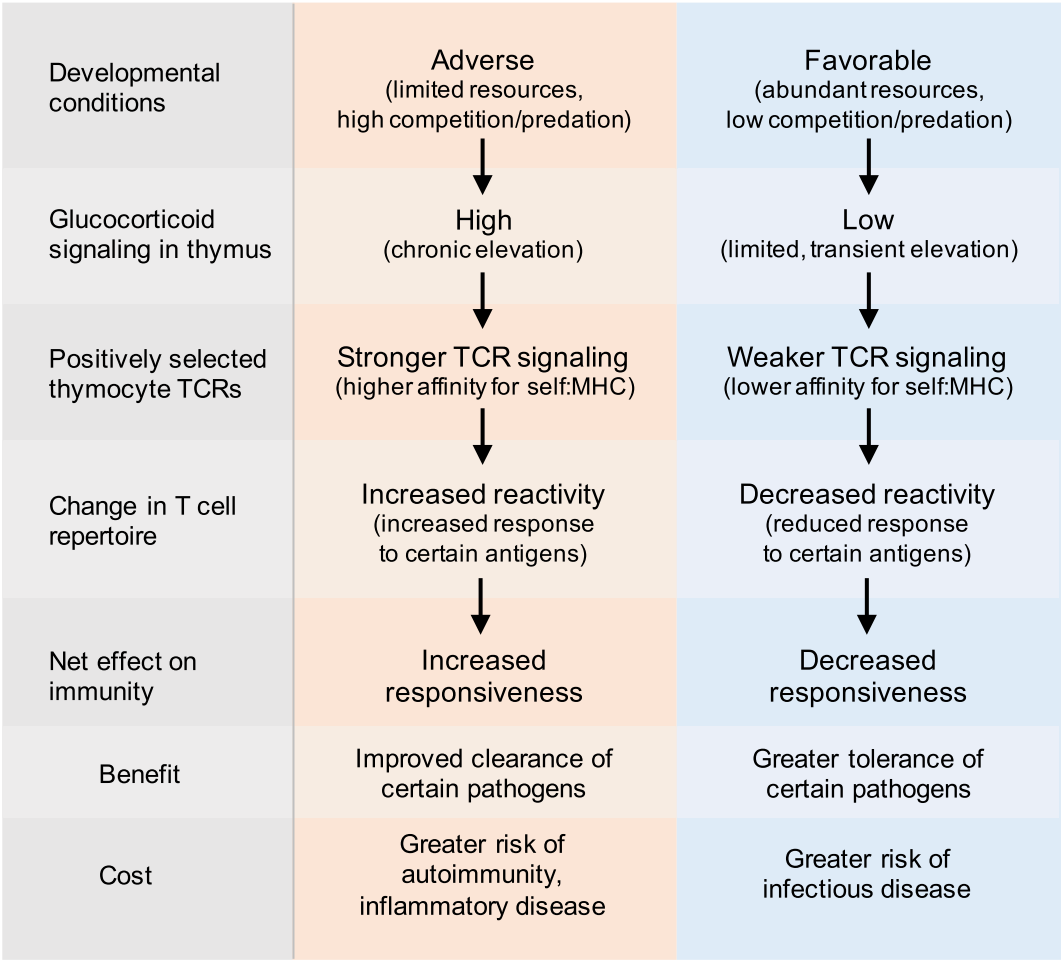

Fig. 7. Hypothetical effects of environmental conditions, especially during development, on T cell development and resulting immune tradeoffs. Adverse conditions, by increasing circulating glucocorticoid and glucocorticoid metabolite levels in the blood, can increase glucocorticoid levels in the thymus. Chronically elevated thymus glucocorticoid concentrations result in positive selection of T cells whose T cell antigen receptors (TCRs) have higher affinity for self-antigen:MHC, and thus are more likely to recognize and signal in response to other MHC-presented antigens (referred to above as "increased reactivity"). The result is a greater antigen-specific T cell-mediated immune response (referred to above as "increased responsiveness") to many different antigens. Notably, lower glucocorticoid levels in the thymus could result in positive selection of TCRs that do not survive under higher glucocorticoid levels, potentially resulting in increased responsiveness to a distinct set of antigens.

Blas et al., 2007; Schmidt et al., 2012), how local steroid production occurs in different tissues (Balthazart et al., 2001; Holloway and Clayton, 2001; London et al., 2006), and how these mechanisms regulate adaptive tradeoffs (Spencer et al., 2005). Moreover, the study of B cell development is simplified by the presence of an organ, the bursa of Fabricius, which is dedicated to this function (Glick et al., 1956; Ratcliffe, 2006). By comparison, the bone marrow serves a large number of different functions. Additionally, the bursa is relatively accessible for experimental manipulations, including removal (bursectomy) (Glick, 1983). Further, agents administered on the cloaca are taken up into the bursa (Sorvari et al., 1975; Paramithiotis and Ratcliffe, 1993; Berghof et al., 2013), which can allow localized administration of drugs (such as inhibitors of GC synthesis or regeneration) and cell labeling *in vivo*. This could then be a means to test GC functions in avian B cell development, and its effects on B cell production, which could be done in both captive and wild birds.

More broadly, variability in local GC production and local GC identities across species indicates that plasma GCs do not necessarily give a representative picture of GC signaling within body tissues, especially lymphoid tissues. It also suggests that local GC production confers some adaptive benefits to the organism. While systemic GC elevation is extremely effective at coordinating an organismal response, systemic GC elevation also comes with many costs – suppression of reproduction, protein synthesis, tissue repair, and immune responses (Sapolsky et al., 2000). Chronically, this can result in severely detrimental effects on health and survival. A benefit then, of local GC production, is that it can allow high GC concentrations to be achieved in specific compartments (such as the thymus) where they are needed, while avoiding detrimental effects on other tissues that would occur with systemic GC secretion. Both local GC synthesis and regeneration, in addition to differential receptor expression (Lattin et al., 2015), could contribute to increased tissue-specific GC signaling when circulating GCs are low, such as in early development of altricial offspring (Schmidt et al., 2003; Wada et al., 2009) or during molt (Newman et al., 2008). GC regeneration could also be useful if GCs and GC metabolites in blood are elevated, by conferring higher GC sensitivity in lymphoid tissues expressing 11β-HSD1. In this way, cellular expression of 11β-HSD1 could function similarly to high levels of GR expression, as both result in increased GC signaling. Activity of 11β-HSD1 might also be suitable for rapid regulation, by changes in cofactor availability or phosphorylation state. This depends on circulating metabolites such as DHC, however, and data on circulating DHC are minimal in mammals (and absent in birds). Local production of cortisol in corticosterone-dominant species could also be beneficial. These two GCs could induce different signaling responses, either *via* distinct signal pathways (Schmidt et al., 2010) or different induction of the same pathways (Garvy et al., 1993). Such differential actions would allow further tissue-specific GC responses. Lymphoid-produced cortisol entering the circulation could also have reduced avoid off-target effects on the brain, compared to corticosterone, as cortisol is selectively excluded from the mouse brain by P-glycoprotein (Karssen et al., 2001).

6. Conclusions

Studies in laboratory mice have clearly demonstrated that lymphoid organs produce GCs and that locally-produced GCs are instrumental in T cell development. Comparative studies in birds have shown that local GC production by lymphoid organs is not limited to mammals and that lymphoid GC levels can vary greatly across species and contexts. Additionally, studies in birds suggest a role for locally-produced GCs in the development of B cells, which mature in the bursa of Fabricius. However, the ways in which lymphoid GCs are regulated, how they affect B cell development, and how they affect immunity and fitness in free-living animals are important topics for future studies. Some questions arising from the current findings include:

1. How do local *versus* adrenal GCs regulate T cell development and repertoire?
2. Do lymphoid GCs play a role in the development of B cells or other leukocytes?
3. Are GCs produced in lymphoid tissues across vertebrate species?
4. How does GC synthesis or regeneration contribute to local GC levels *in vivo*?
5. How do environmental factors regulate lymphoid GCs?
6. Do GCs adaptively program antigen-specific immunity?

Work with a range of species using various measures of antigen-specific immunity will be important to test the contributions of local and systemic GCs to lymphocyte development and whether locally-produced GCs function as regulators of plasticity in adaptive immunity.

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