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Lymphoid organs of neonatal and adult mice preferentially produce active glucocorticoids from metabolites, not precursors

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ABSTRACT

Glucocorticoids (GCs) are circulating adrenal steroid hormones that coordinate physiology, especially the counter-regulatory response to stressors. While systemic GCs are often considered immunosuppressive, GCs in the thymus play a critical role in antigen-specific immunity by ensuring the selection of competent T cells. Elevated thymus-specific GC levels are thought to occur by local synthesis, but the mechanism of such tissue-specific GC production remains unknown. Here, we found metyrapone-blockable GC production in neonatal and adult bone marrow, spleen, and thymus of C57BL/6 mice. This production was primarily via regeneration of adrenal metabolites, rather than *de novo* synthesis from cholesterol, as we found high levels of gene expression and activity of the GC-regenerating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), but not the GC-synthetic enzyme CYP11B1. Furthermore, incubation with physiological concentrations of GC metabolites (11-dehydrocorticosterone, prednisone) induced 11β-HSD1- and GC receptor-dependent apoptosis (caspase activation) in both T and B cells, showing the functional relevance of local GC regeneration in lymphocyte GC signaling. Local GC production in bone marrow and spleen raises the possibility that GCs play a key role in B cell selection similar to their role in T cell selection. Our results also indicate that local GC production may amplify changes in adrenal GC signaling, rather than buffering against such changes, in the immune system.

1. Introduction

Glucocorticoids are steroid hormones synthesized by the adrenal glands that circulate through the blood to coordinate organismal physiology. Circulating glucocorticoids are especially responsive to psychological and physiological stressors, and have pleiotropic effects on neural, metabolic, and immune function (Sapolsky et al., 2000). Acute, transient elevation of circulating glucocorticoids has positive effects on neural and immune function, enhancing cognition and memory (Lupien et al., 2009) and mobilizing immune cells to better respond to pathogens (Bowers et al., 2008), enhancing the ability to survive adverse conditions. In contrast, chronic elevation of circulating glucocorticoids has harmful effects on cognition and mental health (Lupien et al., 2009), suppresses immune responses to pathogens (Dhabhar, 2009), and induces lymphocyte apoptosis (Ashwell et al., 2000). This lymphocyte susceptibility to glucocorticoid-induced apoptosis is clearly demonstrated by the notable reduction in thymus and spleen masses in response to sustained stressors (Selye, 1936; Jellinck et al., 1997).

While circulating, adrenal-derived glucocorticoids act on tissues throughout the body, some tissues can locally produce their own glucocorticoids, allowing tissue-specific regulation of glucocorticoid concentrations and signaling (Taves et al., 2011a). Such local glucocorticoid production is best studied in the thymus, where glucocorticoids antagonize signaling through the T cell antigen receptor (TCR) (Iwata et al., 1991; Vacchio et al., 1994; Van Laethem et al., 2001) and are critical for the selection of immunocompetent T cells (Lu et al., 2000; Mittelstadt et al., 2012). Interestingly, the thymus

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expresses all of the upstream enzymes required for *de novo* conversion of cholesterol into active glucocorticoids (Vacchio et al., 1994; Pazirandeh et al., 1999; Fig. 1). Enzyme activity has also been shown in the thymus, via metyrapone-blockable glucocorticoid production (Vacchio et al., 1994; Lechner et al., 2000; Fig. 1). As metyrapone is commonly used as an inhibitor of the glucocorticoid-synthetic enzyme CYP11B1 (P450c11B1), these data together have led to the conclusion that the thymus produces glucocorticoids from upstream precursors (hereafter, "synthesis"), and that this activity decreases with age (Taves et al., 2011a).

However, more recent work has shown that the thymus also expresses 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), an enzyme that converts inactive glucocorticoid metabolites [11-dehydrocorticosterone (DHC), and cortisone] into active glucocorticoids (Nuotio-Antar et al., 2006; Qiao et al., 2008; Fig. 1). Like upstream CYP11B1, 11β-HSD1 and 11β-HSD2 are also inhibited by metyrapone (Sampath-Kumar et al., 1997; Hostettler et al., 2012). These data raise the possibility that metyrapone-blockable glucocorticoid production might also involve thymus production of glucocorticoids from metabolites (hereafter, "regeneration"), rather than synthesis from precursors.

Glucocorticoid synthesis is independent of circulating steroids (Schmidt et al., 2008), while glucocorticoid regeneration is instead dependent on circulating adrenal-derived glucocorticoid metabolites (Chapman et al., 2013) that can change acutely in response to stressors (Obut et al., 2009) or chronically in psychological disorders (Weber et al., 2000). Thus, the relative contributions of CYP11B1 and 11β-HSD1 could determine how strongly environmental conditions and stressors influence T cell selection. Furthermore, we have previously measured endogenous glucocorticoid levels in developing and adult mice, and have found that in addition to the thymus, glucocorticoids are also locally elevated in the developing bone marrow and spleen (Taves et al., 2015). This indicates that local glucocorticoid production occurs *in vivo*, and that in addition to being critical in T cell development, it may also be important in the development and differentiation of other hematopoietic cells, such as B cells (Gruver-Yates et al., 2013), myeloid cells (Schaer et al., 2002; Trottier et al., 2008), and erythrocytes (Bauer et al., 1999).

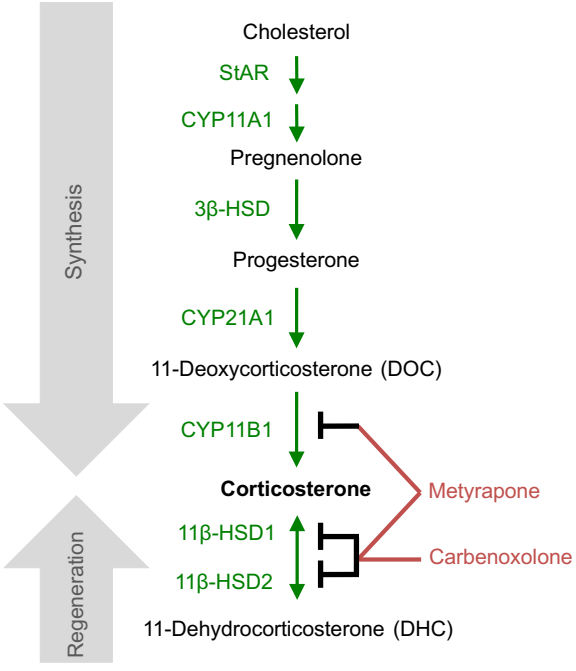

Fig. 1. Simplified glucocorticoid-metabolic pathway. Steroid names are in black, steroidogenic enzyme names and their activities are shaded in green, and the enzyme inhibitors (metyrapone and carbenoxolone) are shaded in red. Here, we define precursor (DOC) conversion to corticosterone as "synthesis," and metabolite (DHC) conversion to corticosterone as "regeneration." The combined effects of synthesis and regeneration are referred to as "production". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Here, we investigated whether murine bone marrow, spleen, and thymus locally express glucocorticoid-metabolic enzymes, and whether this changes with age. We also examined whether glucocorticoid production ("production" referring to both synthesis and regeneration) occurs via synthesis from upstream precursors, or via regeneration of metabolites. As glucocorticoid precursors are elevated in neonatal lymphoid organs (Taves et al., 2015), we hypothesized that neonatal lymphoid organs would produce glucocorticoids via synthesis, and that adult lymphoid organs would have decreased synthesis and increased regeneration. Finally, as a functional test of glucocorticoid production, we quantified glucocorticoid receptor-mediated lymphocyte apoptosis in the presence of physiologically relevant substrate concentrations.

2. Materials and methods

2.1. Study 1: Endogenous gene expression of glucocorticoid-metabolic enzymes

2.1.1. Subjects

Female and male C57BL/6 mice were bred and housed at the Centre for Disease Modeling, a specific pathogen-free facility at the University of British Columbia. Mice were group housed with corncob bedding, under a 14:10 light:dark cycle, with *ad libitum* water and food (Teklad, diet 2918 for adults, diet 2919 for breeders). All protocols were approved by the UBC Animal Care Committee (A12-0119). All tissue samples were collected in the morning (between 0800 and 1200 h), to reduce possible diurnal variation in steroid levels.

2.1.2. Tissue collection

Neonates (5 days old, with day 0 being the morning that pups were first present) or adults (2–3 months old) were deeply anesthetized with isoflurane in oxygen (<2 min) and euthanized by rapid decapitation. Decapitation and trunk blood collection was completed in less than 3 min after initial disturbance. Immediately after euthanasia, spleen and thymus were collected, cleaned of fat and connective tissue, and bone marrow was flushed from femurs with ice-cold PBS. Tissues were then snap-frozen on dry ice and stored at −80 °C.

2.1.3. Endogenous glucocorticoid concentrations

This work was performed using the same mouse line as in our previous work (Taves et al., 2015), but our previous animal facility (Wesbrook Animal Unit) was closed and the mouse colony was rederived in the Centre for Disease Modeling (detailed in Taves (2015)). Therefore, we conducted a pilot study, to test whether neonates in this facility exhibited similar local elevation of lymphoid glucocorticoid levels, as previously reported (Taves et al., 2015). We again found that progesterone and corticosterone concentrations were locally elevated in neonatal lymphoid organs, compared with concentrations in circulating blood. However, unlike our previous work, we did not detect high concentrations of cortisol. Thus, we focused this series of experiments on corticosterone, which exhibited highly similar patterns of local elevation in lymphoid organs of neonates from both animal facilities.

2.1.4. Real-time quantitative PCR (qPCR)

Samples were homogenized in ice-cold isol-RNA lysis reagent (5 Prime 2302700), and RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% ethanol (Taves et al., 2015). Complementary DNA was reverse-transcribed from 5 μg of total RNA with a Maxima First Strand Synthesis kit (Thermo K1641), and qPCR for gene expression of steroid-metabolic enzymes was performed in triplicate with 5′ nuclease probe-based assays (Integrated DNA Technologies, details in Supplementary Table 1). These assay probes contain a 5′ fluorescent reporter, and internal and 3′ fluorescence quenchers. Primer extension cleaves the probe, separating the reporter and quenchers, resulting in emission of a target-specific fluorescent signal. Quantitative PCR was performed with Brilliant III QPCR mastermix (Agilent, 600880), run at 95 °C for 3 min, followed by 45 cycles of 95 °C for 5 s and 60 °C for 10 s. All detectable samples had Cq (quantification cycle) values less than 40 cycles. Rps29, Rpl4, and Oaz1 were used as reference genes (de Jonge et al., 2007), with the mean of these three Cq values used as the reference value. The use of three or more reference genes greatly improves the reliability of gene expression data (Vandesompele et al., 2002). Importantly, all assays for a single sample were run on the same plate, to allow analysis of the relative expression of different steroidogenic enzymes. Expression of genes of interest were expressed relative to that of adult thymus Cyp11b1. Negative controls (no RNA or no reverse transcription) were always undetectable.

2.2. Study 2: Ex vivo glucocorticoid production

2.2.1. Tissue culture

Subjects were anesthetized and euthanized as described above, and the thymus, spleen, and femurs were collected. Bone marrow was flushed from femurs with ice-cold culture media, and thymus and spleen were minced in culture media consisting of RPMI 1640 (Hyclone SH3009601) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone SH3006802), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 12 μM L-glutamine. All tissues were dispersed with a pipette, and incubated for 24 h in 5% CO_2 at 37 °C in 4 ml medium containing vehicle (0.2% ethanol), 0.9 mM metyrapone (Vacchio and Ashwell, 1997; Cima et al., 2004; Tocris), or 1 μM DOC or DHC steroid substrate (Vukelic et al., 2011; Steraloids) (Fig. 1). For each adult subject, tissues were split in half; one half treated with vehicle and the other half treated with metyrapone or steroid substrate, allowing for within-subject analysis. Tissues from neonates were not split due to the small size. After 24 h, adherent cells were loosened with a pipet, and cells and supernatant were transferred into a conical tube. In some experiments, 1 ml of methanol was immediately added and samples were frozen at −80 °C, while in other experiments, samples were spun for 6 min at 500 × g and 1 ml of methanol added to the supernatant before being frozen at −80 °C.

2.2.2. Steroid extraction

Steroids were extracted from samples using solid phase extraction (SPE) with C_{18} columns as previously described (Newman et al., 2008; Taves et al., 2010). SPE columns (Agilent, cat. 12113045) were primed with 3 ml methanol, equilibrated with 10 ml water, and tissue culture samples were thawed, diluted with 5 ml of water, and loaded onto columns. Samples were washed with 10 ml 40% methanol to remove interfering substances (Brummelte et al., 2010; Taves et al., 2011b), and unconjugated steroids were eluted with 5 ml 90% methanol and dried in a vacuum centrifuge (ThermoElectron SPD111V).

2.2.3. Steroid separation

When tissues were cultured in the presence of exogenous steroid substrate (DOC or DHC), extracted steroids were further processed using reversed-phase HPLC to avoid any antibody cross-reactivity (Gilson 322 pump, Gilson FC 204 fraction collector), based on a previously published protocol (Taves et al., 2015, 2016). A Waters SymmetryShield C_{18} column (4.6 × 250 mm, 5 μm silica particles) kept at 40 °C was used for steroid separation. The mobile phases were 0.01% formic acid in water (Solvent A) and 0.01% formic acid in acetonitrile (Solvent B). Following SPE, steroid residues were resuspended in 45 μl 20% acetonitrile, 0.01% formic acid in water, centrifuged at 16,000*g* for 5 min, and supernatants transferred to HPLC vials. From each sample, 16 μl was loaded onto the HPLC column and eluted at a flow rate of 1.0 ml/min. From 0 to 15 min the concentration of Solvent B increased from 20% to 45%, and from 15 to 19 min the concentration of Solvent B increased from 45% to 100%. The Solvent B concentration was maintained at 100% until 25 min, when it was decreased back to 20% and the column was equilibrated until 35 min. The gradients and steroid elution times are given in Supplementary Fig. 1. This relatively long run time ensured clean separation of our steroids of interest. Eluting corticosterone was collected in a 0.45 min fraction (from 17.47 to 17.92 min), which was dried in a vacuum centrifuge. To further ensure that there was no sample carry-over, a stringent cleaning protocol was used between samples, which included washing the column with 100% Solvent B for 15 min, rinsing the fraction collector with 100% Solvent B for 4 min, and rinsing the injection needle, syringe, and sample loop with 50% Solvent B.

2.2.4. Immunoassay

Glucocorticoids were quantified after SPE or after SPE + HPLC using specific and sensitive immunoassay kits (corticosterone radioimmunoassay from MP Biomedicals, cortisol enzyme immunoassay from Salimetrics) as previously described (Taves et al., 2015, 2016).

2.2.5. Liquid chromatography–tandem mass spectrometry

Although HPLC separation resulted in clear separation of corticosterone from major precursors and metabolites, we wanted to ensure that our measurements were not affected by any cross-reactivity to unknown co-eluting steroids. Therefore, we quantified corticosterone in our HPLC fractions using LC–MS/MS, as previously described (Taves et al., 2015). Dried HPLC fractions were resuspended and injected into a Shimadzu Prominence LC20AC system, separated using a Phenomenex Gemini NX-C18 column, and corticosterone detected with multiple reaction monitoring (MRM), with two MRM transitions (quantifier m/z 347.4 → 121.0 and qualifier m/z 347.4 → 97.1) on an AB Sciex 5500 Qtrap triple quadrupole tandem mass spectrometer in positive electrospray ionization mode.

2.3. Study 3: Lymphoid-derived glucocorticoid induction of apoptosis

2.3.1. Cell culture, caspase activity, and flow cytometry

Adult mouse tissues were collected into ice-cold PBS, cells dispersed through a 70 μm mesh, erythrocytes lysed with ammonium-chloride-potassium lysis buffer (bone marrow and spleen only), and remaining cells washed and resuspended in culture medium. Cells from the same tissue were then aliquoted into different wells containing DOC (100 nM), DHC (100 nM), dexamethasone (100 nM), prednisone (100 nM), metyrapone (0.9 mM), carbenoxolone, an inhibitor of 11β-HSD1 and 11β-HSD2 (1 mM), RU-486, a glucocorticoid receptor antagonist (1 μM), or a combination of these. Cells were incubated for 24 h in 5% CO_2 at 37 °C as above. For some experiments, culture supernatants (100 μl) were then transferred to a fresh tube, diluted in 1 ml methanol, and

stored at −80 °C (for quantification of steroids). Cells were washed and incubated for 30 min in RPMI 1640 with CellEvent Caspase 3/7 Detection Reagent (Life Technologies, C10427), then washed in FACS buffer and stained with labeled monoclonal antibodies against CD4, CD8, and B220 for 30 min on ice (Supplementary Table 2). Samples were then resuspended with 7-AAD (7-aminoactinomycin D) or DAPI (4′,6-diamidino-2-phenylindole), which both stain cells with compromised plasma membranes, to determine cell viability. Data were acquired on a FACSCanto or LSR II flow cytometer (BD Biosciences), and analysis was conducted using FlowJo software (Tree Star).

2.4. Statistical analysis

Undetectable steroid sample values (those below the lowest standard on the standard curve or those lower than the average water blank + two standard deviations, whichever was greater) were set to zero. Log-transformed data were tested for significant deviation from normality using the Shapiro-Wilk test or Mauchly's test, and analyzed using linear mixed-effects models in R (R Core Team, 2005) and Prism 5. Planned comparisons were conducted using paired *t*-tests, unpaired *t*-tests, or Wilcoxon tests as appropriate. Significance was set at $\alpha = 0.05$, and data are shown as mean ± SEM. Data from females and males were pooled, as no sex differences were detected.

3. Results

3.1. Study 1: Endogenous gene expression of glucocorticoid-metabolic enzymes

3.1.1. Lymphoid organs express high levels of Hsd11b1 but little or no Cyp11b1

To first investigate the potential for lymphoid organ conversion of steroid substrates into active glucocorticoids, we quantified gene expression of glucocorticoid-producing enzymes using highly specific, probe-based qPCR assays. The enzyme CYP11B1 converts the upstream precursor, DOC, into corticosterone, while the enzyme 11β-HSD1 converts the inactive 11-keto metabolite, DHC, into active corticosterone (Chapman et al., 2013). The enzyme 11β-HSD2 catalyzes the reverse reaction, converting active corticosterone into inactive DHC (Chapman et al., 2013).

Across lymphoid organs, gene expression of 11β-HSD1 (coded by the *Hsd11b1* gene) was higher than expression of 11β-HSD2 (*Hsd11b2*), which was in turn higher than expression of CYP11B1 (Fig. 2A–C). In neonates, gene expression of 11β-HSD1 was approximately 1000-fold higher than gene expression of CYP11B1, which was often undetectable. The pattern was similar in adults, where expression of CYP11B1 was usually undetectable, 11β-HSD1 was higher, and 11β-HSD2 was lower, relative to levels in neonates (Fig. 2A–C). Negative controls (no template or no reverse transcriptase) were always undetectable. These data suggest that in neonatal lymphoid organs, glucocorticoid production may occur largely via regeneration from DHC, rather than synthesis from DOC. Furthermore, local glucocorticoid production may actually increase into adulthood, due to increased expression of 11β-HSD1 and reduced expression of 11β-HSD2 (Fig. 2A–C).

3.1.2. Neonatal but not adult lymphoid organs express upstream steroidogenic enzymes needed to synthesize glucocorticoids

We also assayed gene expression of all of the steroidogenic enzymes upstream of CYP11B1. In neonatal tissue, we frequently detected mRNA for all of the enzymes needed for conversion of cholesterol, the precursor of all steroids, into DOC (Supplementary Fig. 2A–C). As CYP11B1 gene expression was lower than that for all other enzymes, low levels of CYP11B1 might be the limiting step in lymphoid glucocorticoid synthesis. In adult lymphoid organs, gene expression of upstream enzymes was mostly reduced, although StAR and CYP21A1 expression increased in the spleen. Expression of 3β-HSD6 (coded by *Hsd3b6*) was undetectable in adult bone marrow and spleen, similar to expression of CYP11B1. Expression of 3β-HSD1 (coded by *Hsd3b1*) was undetectable in all tissues at both ages. These data suggest that glucocorticoid synthesis from precursors could occur in neonates, although gene expression of 11β-HSD1 was generally much higher than that of upstream glucocorticoid-synthetic enzymes. In adult bone marrow and spleen, the absence of 3β-HSD6, 3β-HSD1 and CYP11B1 expression suggests that regeneration is the major pathway for production of glucocorticoids, and that synthesis from precursors is unlikely.

3.2. Study 2: Ex vivo glucocorticoid production

To explore the net direction of glucocorticoid production or metabolism in lymphoid organs, we began by culturing minced tissues. This avoided the selective reduction of certain cellular subsets, such as stromal and dendritic cells, which might be depleted during cell isolation and dispersal, and maintained fragments of intact tissue architecture.

3.2.1. Glucocorticoids are produced by bone marrow, spleen, and thymus, and demonstrate age-related increase

To test whether lymphoid organs are able to actively produce glucocorticoids, we incubated minced tissues in the presence or

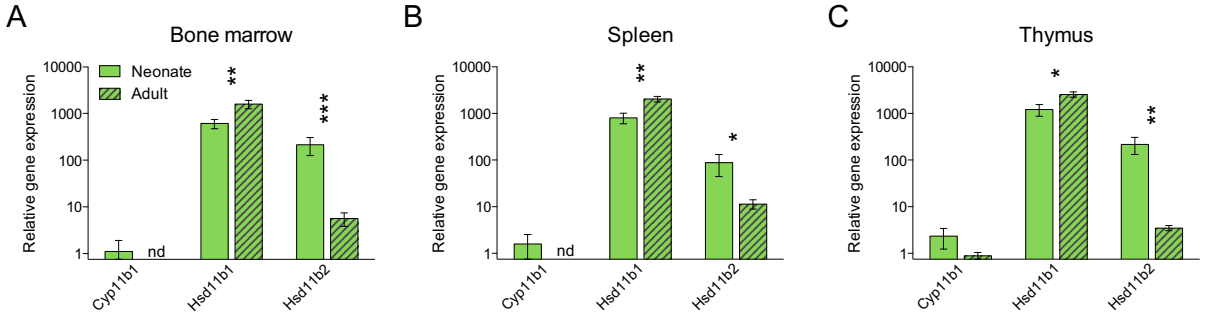

Fig. 2. Lymphoid organ *Hsd11b1* gene expression is much higher than *Cyp11b1* expression, while *Hsd11b2* expression is intermediate. Relative expression was quantified in neonatal and adult (A) bone marrow, (B) spleen, and (C) thymus, using highly specific 5′ nuclease probe-based qPCR assays. Expression was corrected using the mean of three reference genes *Rps29*, *Rpl4*, and *Oaz1*, and relative expression is shown relative to adult thymus *Cyp11b1*. The use of three or more reference genes greatly improves the reliability of gene expression data. Note the log-scale for the y-axes. In some cases, *Cyp11b1* expression was not detectable (nd). Data are presented as means ± SEM, and age-related differences in expression of a particular gene are indicated as follows, $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$. Relative expression was always significantly different across the three genes (*Hsd11b1* > *Hsd11b2* > *Cyp11b1*), at both ages. N = 9 neonates, 8 adults.

absence of metyrapone, which inhibits glucocorticoid synthesis by CYP11B1, regeneration by 11β-HSD1, and inactivation by 11β-HSD2. We then quantified corticosterone and cortisol in extracted culture medium and tissue. While we have previously found locally elevated levels of both corticosterone and cortisol in lymphoid organs of neonatal mice (Taves et al., 2015), here we detected little or no cortisol in any samples (data not shown) and thus focused on corticosterone. We found that in the presence of metyrapone, conditioned media from bone marrow, spleen, and thymus all had dramatically reduced concentrations of corticosterone (Fig. 3A and B), demonstrating net glucocorticoid production in each of these tissues, which could be blocked by inhibition of glucocorticoid-producing enzymes. Consistent with endogenous gene expression data, the magnitude of metyrapone-blockable corticosterone, even after correction for tissue mass, was much higher in adults than in neonates across all tissues (Fig. 3C). This provides further evidence that lymphoid glucocorticoid production occurs throughout life, and is increased in adults. As these experiments were performed with steroid-free medium, these data also show that glucocorticoid production occurs in the presence of an endogenous substrate present in freshly-collected tissues.

3.2.2. Lymphoid organs convert the metabolite DHC but not the precursor DOC into corticosterone

To test whether our gene expression data corresponded with CYP11B1 or 11β-HSD1 enzyme activity, we next incubated tissues in steroid-free medium supplemented with the enzyme substrates, DOC or DHC. Conditioned media samples were extracted, and steroids were separated with HPLC to avoid potential antibody cross-reactivity.

Consistent with our gene expression results, incubation of neonatal tissues with DOC resulted in moderate (bone marrow) or undetectable (spleen, thymus) increases in corticosterone levels compared to paired vehicle samples, while incubation with DHC resulted in dramatic increases in corticosterone levels (Fig. 3D). A

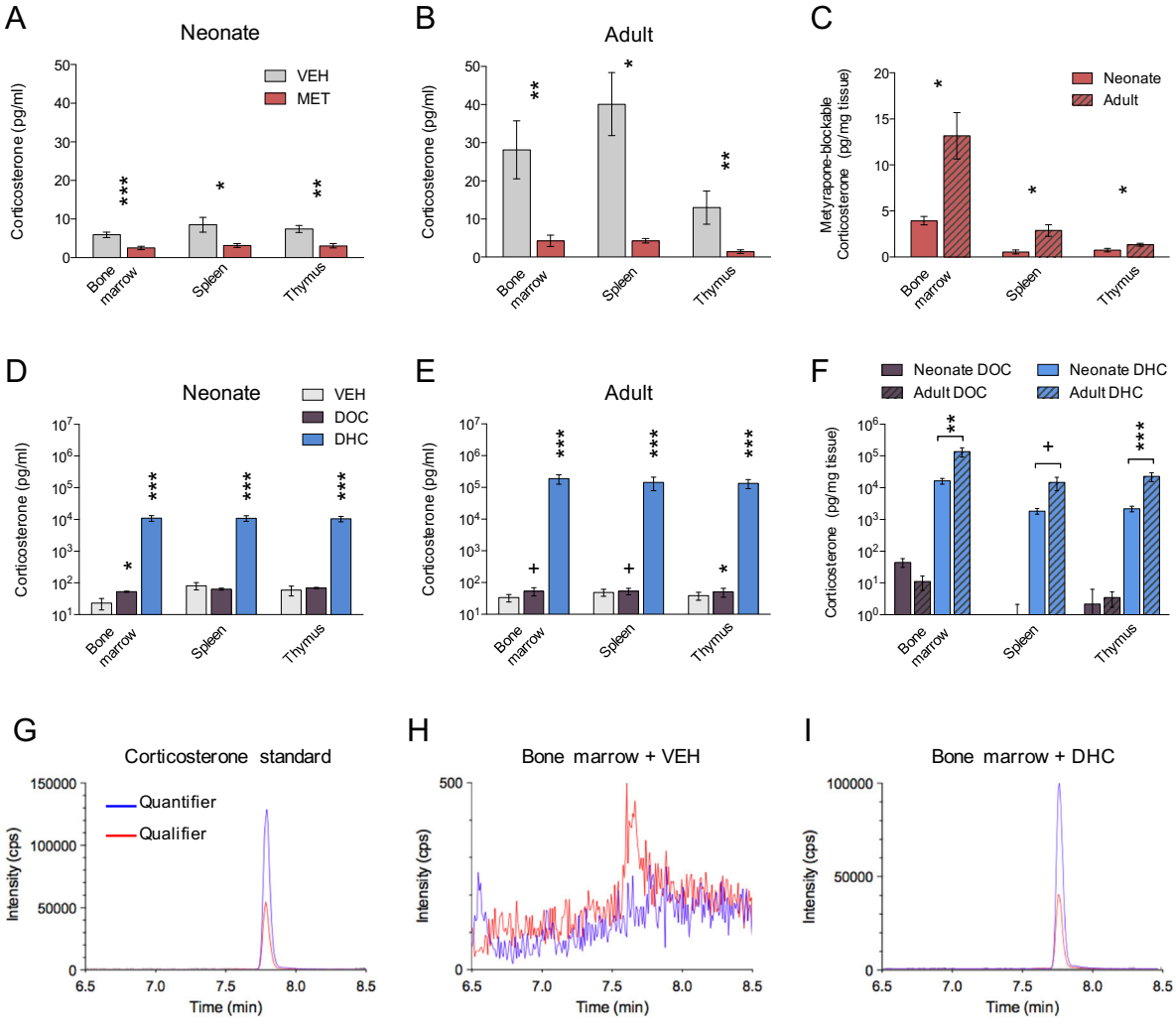

Fig. 3. Glucocorticoid production by bone marrow, spleen, and thymus. (A) Neonatal and (B) adult mouse lymphoid organs were cultured *ex vivo* for 24 h with vehicle (VEH) or 0.9 mM metyrapone (MET), which inhibits CYP11B1, 11β-HSD1, and 11β-HSD2. No exogenous substrate was added. Steroids were extracted using SPE and corticosterone was quantified with a sensitive and specific RIA. (C) Metyrapone-blockable glucocorticoid production was estimated by subtracting corticosterone levels in metyrapone-incubated samples from corticosterone levels in paired vehicle-incubated samples, and correcting for average tissue mass in each sample. (D) Neonatal and (E) adult tissues were cultured *ex vivo* with vehicle, 1 μM of the corticosterone precursor DOC, or 1 μM of the corticosterone metabolite DHC. Steroids were extracted using SPE, corticosterone isolated using HPLC, and quantified via RIA. Note the log scale for the y-axes. (F) DOC and DHC conversion to corticosterone in neonatal and adult lymphoid organs, corrected for average tissue mass in each sample. All data are presented as means ± SEM, and age or treatment differences are indicated as follows, + $p \leqslant 0.10$, $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$. N = 5–7 neonates, 4–6 adults. (G–I) representative LC–MS/MS multiple reaction monitoring (MRM) traces confirming RIA corticosterone measurements. (G) Corticosterone standard, (H) sample from vehicle-incubated adult bone marrow, and (I) sample from DHC-incubated adult bone marrow. Other tissues shown in Supplementary Fig. 3.

similar pattern was observed in adult tissues, where incubation with DOC had little effect on corticosterone levels but incubation with DHC again resulted in large increases in corticosterone levels (Fig. 3E). The corticosterone increase in the presence of DHC was higher with adult tissues than with neonatal tissues, and when corrected for tissue mass, adult bone marrow and thymus had higher corticosterone-producing activity than neonatal bone marrow and thymus (Fig. 3F). Quantification with liquid chromatography-tandem mass spectrometry (LC–MS/MS) closely matched our immunoassay measurements of corticosterone (Fig. 3G–I; Supplementary Fig. 3E–G).

To rule out experimental artifacts, we found that medium containing either DOC or DHC, incubated without tissue and processed in parallel with tissue-containing medium, contained little or no detectable corticosterone. Thus, DOC and DHC did not contaminate the corticosterone HPLC fraction or spontaneously undergo conversion into corticosterone in the absence of tissue. DOC and DHC conversion to corticosterone could be inhibited by coincubation with metyrapone (Supplementary Fig. 3A and B), showing that our original finding of metyrapone-blockable glucocorticoid production was consistent with inhibition of 11β-HSD1 conversion of DHC into corticosterone. The minimal conversion of DOC into corticosterone was specific to lymphoid organs, as adrenal corticosterone production was increased in medium supplemented with DOC (Supplementary Fig. 3C). Furthermore, DHC conversion to corticosterone was blocked by boiling lymphoid tissue before incubation (Supplementary Fig. 3D), showing that conversion requires intact tissue and enzyme function.

3.3. Study 3: Lymphoid-derived glucocorticoid induction of apoptosis

Pilot studies indicated that the large majority of blockable glucocorticoid production occurred in dispersed cells after separation through a 70 μm cell strainer, so we next used dispersed cells to allow equal aliquoting of samples across multiple pharmacological treatments, and to facilitate subsequent single-cell analysis.

3.3.1. T and B lymphocytes are both highly sensitive to glucocorticoid induction of apoptosis

To first test the sensitivity of T and B lymphocytes to glucocorticoid-induced apoptosis, we incubated bone marrow, spleen, and thymus cells from adult mice in the presence of increasing doses of corticosterone (Fig. 4). We then detected induction of apoptosis by measuring Caspase-3/7 activity via flow cytometry, and comparing the proportion of Caspase+ cells in corticosterone-incubated samples to vehicle-incubated samples. In the bone marrow, corticosterone induction of apoptosis was strongest in $CD8^+$ T cells, followed by $CD4^+$ T cells and $B220^{hi}$ (mature) B cells, and weakest in $B220^+$ (immature) B cells. In the spleen, $CD8^+$ T cells, $CD4^+$ T cells, and $B220^+$ B cells were all similarly sensitive. In the thymus, all subsets were sensitive, although DN ($CD4^-CD8^-$ double negative) and $CD8^+$ thymocytes had a detectable increase in apoptosis at a lower corticosterone dose than DP ($CD4^+CD8^+$ double positive) and $CD4^+$ thymocytes.

3.3.2. Dehydrocorticosterone induction of apoptosis is dependent on both 11β-HSD1 activity and glucocorticoid receptor binding

As a functional test of lymphoid 11β-HSD1 activity, we incubated bone marrow, spleen, or thymus cells in the presence of 100 nM DHC, which is at the high end of physiological DHC concentrations in rodent plasma (Obut et al., 2004, 2009) and cortisone concentrations in human plasma (Nomura et al., 1997; Kushnir et al., 2004). Samples were also incubated with carbenoxolone, an inhibitor of 11β-HSD1 and 11β-HSD2, or RU-486, a glucocorticoid receptor antagonist. Similar to our results with minced tissues, corticosterone levels were dramatically increased in the presence of DHC, and reduced in the presence of carbenoxolone (Fig. 5A). We also found that DHC potently induced apoptosis in T and B cells, and that this was blocked by either 11β-HSD1 inhibition with carbenoxolone or glucocorticoid receptor antagonism with RU-486 (Fig. 5B–D; Supplementary Fig. 4). DHC-induced apoptosis exhibited a dose-dependent increase, and there was clear induction of apoptosis after 4 and 24 h, although greater after 24 h (data not shown). The effect of carbenoxolone was also dose-dependent, with lower concentrations exhibiting lower inhibition of DHC-induced apoptosis (data not shown). DOC was less effective than DHC at inducing apoptosis (Supplementary Fig. 4). Thus, lymphoid regeneration of glucocorticoids is sufficient to potently induce glucocorticoid receptor-mediated induction of lymphocyte apoptosis. The DHC-induced changes in proportions of apoptotic T and B cells largely mimicked the patterns seen with corticosterone (Fig. 4).

3.3.3. Prednisone- but not dexamethasone-induced apoptosis is blocked by inhibition of 11β-HSD1

Finally, to test whether expression of 11β-HSD1 might play a role in determining lymphoid susceptibility to different clinically-used glucocorticoids, we incubated lymphoid cells with 100 nM prednisone or dexamethasone, which is within the clinically relevant range of these steroids (Shibasaki et al., 2007; Yang et al., 2008). Samples were also incubated in the presence or absence of carbenoxolone. Apoptosis induction by prednisone,

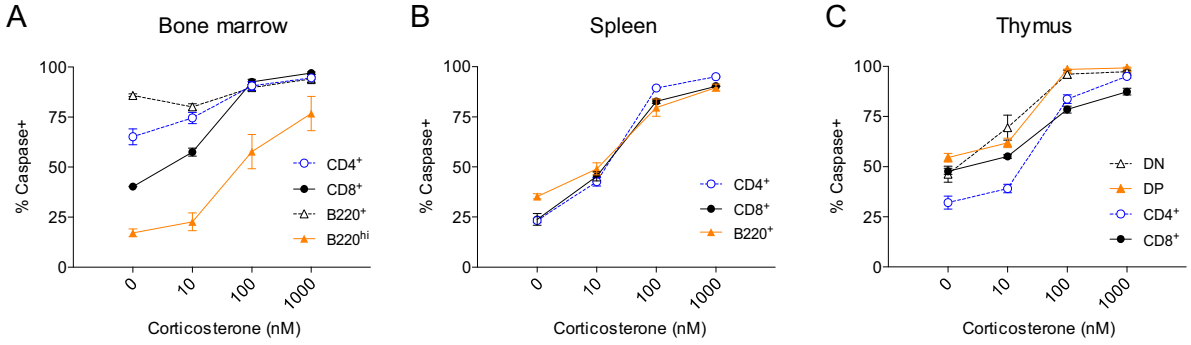

Fig. 4. Corticosterone induction of apoptosis in T and B lymphocytes in (A) bone marrow, (B) spleen, and (C) thymus of adult mice. Cells were cultured for 24 h in the indicated concentrations of corticosterone, and Caspase 3/7 activity was used to detect induction of apoptosis. Data are expressed as the percent of cells exhibiting Caspase activity. Relative sensitivity to corticosterone-induced apoptosis was compared by determining the dose at which the percent of Caspase$^+$ cells was significantly different from that in vehicle-treated cells. Corticosterone sensitivity in bone marrow was $CD8^+ > CD4^+ = B220^{hi} > B220^+$, and in thymus was $DN = CD8^+ > DP = CD4^+$. Spleen lymphocytes were all similarly sensitive to corticosterone.

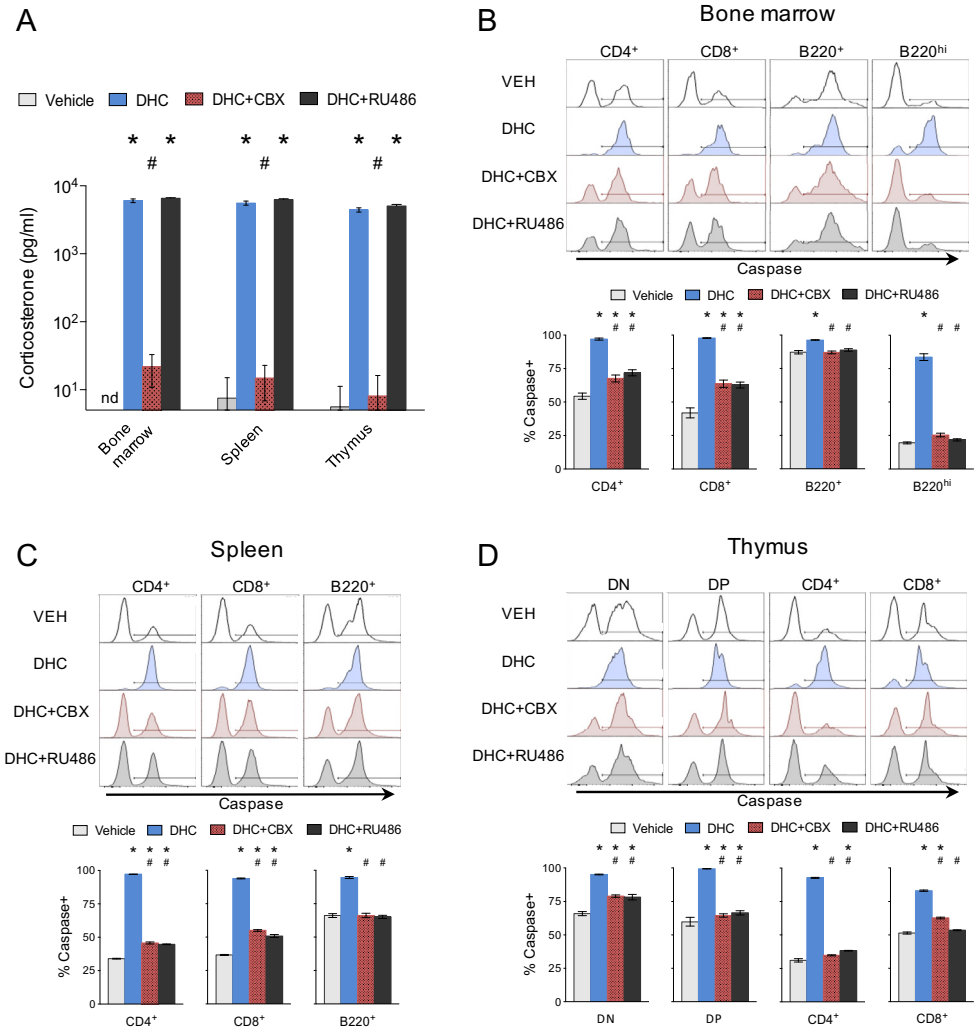

Fig. 5. DHC-induced apoptosis of T and B lymphocytes is dependent on 11β-HSD1 activity and glucocorticoid receptor binding. Bone marrow cells, splenocytes, and thymocytes from adult mice were cultured for 24 h in the presence of vehicle (VEH) or 100 nM 11-dehydrocorticosterone (DHC), with 1 mM carbenoxolone (CBX, an 11β-HSD inhibitor) or with 1 μM RU-486 (a glucocorticoid receptor antagonist). (A) An aliquot of culture supernatant was processed with SPE and HPLC prior to RIA corticosterone quantification. (B–D) Caspase 3/7 activity was used to detect induction of apoptosis, and is expressed as the percent of cells exhibiting Caspase 3/7 activity. An * indicates a significant difference from vehicle-treated samples, a # indicates a significant difference from DHC-treated samples, and "nd" indicates samples were not detectable. FACS plots and DOC, CBX, and RU486 data are given in Supplementary Fig. 4.

which is inactive until converted into active prednisolone by 11β-HSD1, was inhibited by carbenoxolone (Fig. 6, Supplementary Fig. 5). In contrast, apoptosis induction by dexamethasone, an active glucocorticoid receptor ligand, was unaffected by carbenoxolone (Supplementary Fig. 5). These results show that lymphoid 11β-HSD1 converts clinically relevant levels of prednisone into active prednisolone, increasing lymphoid responsiveness to prednisone.

4. Discussion

Here, we show that murine bone marrow, spleen, and thymus are each able to produce glucocorticoids from endogenous substrates, and that this occurs primarily via glucocorticoid regeneration by 11β-HSD1, rather than synthesis by CYP11B1. We also unexpectedly found that lymphoid corticosterone production was higher in adult than in neonate lymphoid organs, instead of decreasing with age. These findings, together with 11β-HSD1- and glucocorticoid receptor-dependent DHC induction of lymphocyte apoptosis, indicate that 11β-HSD1 regeneration of glucocorticoids is likely a determinant of thymocyte survival thresholds in T cell ontogeny, and is likely also important in lymphoid development and function in the bone marrow and spleen. Furthermore, these data indicate that lymphoid organs may be preferentially susceptible to 11β-HSD1-dependent synthetic glucocorticoids, compared to tissues with little or no 11β-HSD1.

4.1. Bone marrow, spleen, and thymus produce glucocorticoids

Glucocorticoids potently regulate the activity of lymphocytes (Ashwell et al., 2000) and other leukocytes (Tuckermann et al., 2007), and this is generally assumed to follow systemic patterns of glucocorticoid synthesis by the adrenal glands. However, we previously found that endogenous glucocorticoid concentrations were locally elevated in murine lymphoid organs, especially in early life when circulating adrenal-derived glucocorticoid concentrations were low (Taves et al., 2015). Here, we found that the bone marrow and spleen, like the thymus, locally produce glucocorticoids, and thus also have local control of glucocorticoid signaling rather than merely functioning as passive recipients of circulating adrenal glucocorticoids. Unexpectedly, lymphoid glucocorticoid production increased with age, suggesting that local glucocorticoid

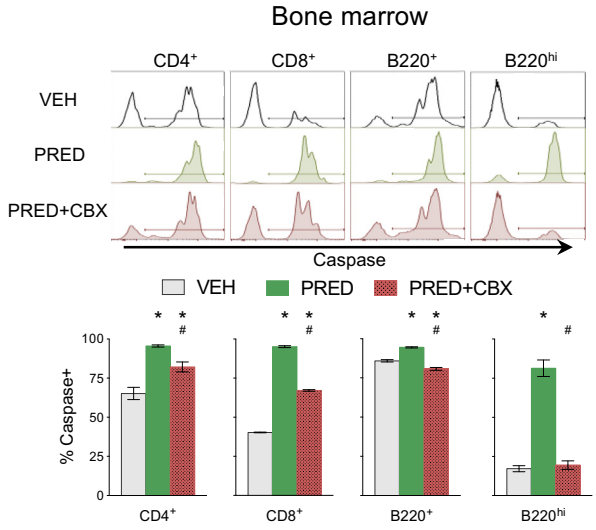

Fig. 6. Prednisone-induced apoptosis of T and B lymphocytes is dependent on 11β-HSD1 activity. Total bone marrow cells from adult mice were cultured for 24 h in the presence of vehicle (VEH) or 100 nM prednisone (PRED), which is dependent on 11β-HSD-mediated conversion to prednisolone to bind the glucocorticoid receptor, and with or without 1 mM carbenoxolone (CBX). Caspase 3/7 activity was used to detect induction of apoptosis, and is expressed as the percent of cells exhibiting Caspase 3/7 activity. An * indicates a significant difference from vehicle-treated samples, and a # indicates a significant difference from PRED-treated samples. Patterns were similar in the spleen and thymus (Supplementary Fig. 5). In contrast, dexamethasone-induced apoptosis in bone marrow, spleen, and thymus was not reduced by CBX (Supplementary Fig. 5).

production is not only important when systemic glucocorticoid levels are minimal or absent (Schmidt et al., 2008). Local glucocorticoid production may instead regulate the development and function of lymphocytes and other hematopoietic cells throughout life.

The role of locally-regenerated glucocorticoids in the bone marrow and spleen is unclear, but may parallel its role in the thymus, which is better studied. In the thymus, glucocorticoids antagonize TCR signaling (Iwata et al., 1991; Vacchio et al., 1994, 1999; Vacchio and Ashwell, 1997; Van Laethem et al., 2001) and ensure the selection and continued development of competent T cells (Mittelstadt et al., 2012). Thymocyte 11β-HSD1 expression may be highest in the cortico-medullary junction and medulla, consistent with a role in negative selection (Nuotio-Antar et al., 2006). In the spleen, TCR signaling increases $CD4^+$ T cell expression of 11β-HSD1 (Zhang et al., 2005), and increased glucocorticoids antagonize TCR signaling (Jamieson and Yamamoto, 2000) while upregulating expression of the IL-7 receptor, which is critical for T cell development and survival (Lee et al., 2005). Thus, a TCR signaling-mediated increase in 11β-HSD1 activity may function as a feedback loop to control T cell activation and survival in the spleen, and also T cell activation and survival in the bone marrow.

Analogous to their role in T cell signaling, locally-produced glucocorticoids in the bone marrow and spleen might also function in B cell development, ensuring the production of an appropriately reactive B cell repertoire. Consistent with this, immature B cell subsets in the bone marrow have especially high glucocorticoid receptor levels (Gruver-Yates et al., 2013), and glucocorticoids upregulate IL-7 receptor expression (Shibata et al., 2007). Furthermore, the avian bursa of Fabricius, an organ dedicated to B cell development (Glick, 1956), also produces high local levels of glucocorticoids (Lechner et al., 2001; Schmidt et al., 2008; Taves et al., 2016). Together, these data indicate that B cells are a target of locally-produced glucocorticoids in the mammalian bone marrow and spleen. Other potential targets include macrophages (Zhang and Daynes, 2007), dendritic cells (Freeman et al., 2005), and neutrophils (Kardon et al., 2008), which all express 11β-HSD1.

4.2. Glucocorticoid regeneration versus synthesis

We have previously found that upstream glucocorticoid precursors are locally elevated in parallel with glucocorticoids in developing bone marrow, spleen, and thymus (Taves et al., 2015). Additionally, in the thymus, other studies have found activity of upstream glucocorticoid-synthetic enzymes (Vacchio et al., 1994; Pazirandeh et al., 1999; Lechner et al., 2000). Thus, we expected that corticosterone would be synthesized as an "immunosteroid" from upstream precursors, via DOC (Schmidt et al., 2008). However, we found that gene expression and activity of 11β-HSD1 were approximately 1000-fold higher than expression and activity of CYP11B1, which were often undetectable. This large difference indicates that our observed metyrapone-blockable corticosterone production is primarily due to 11β-HSD1 regeneration from endogenous DHC. Our data also show that glucocorticoid regeneration by 11β-HSD1 predominates over inactivation by 11β-HSD2, which is also inhibited by metyrapone. Together, our data show that 11β-HSD1 is the major contributor to lymphoid glucocorticoid production.

In the bone marrow and spleen, this finding is consistent with previous reports of 11β-HSD1 (in osteoblasts, Cooper et al., 2002; in splenocytes, Zhang et al., 2005), but to our knowledge this is the first study showing that the net direction of glucocorticoid metabolism in these tissues is glucocorticoid production. However, our results appear to conflict with previous work on glucocorticoid production in the thymus. While several studies have examined mouse thymus glucocorticoid synthesis, only one has specifically demonstrated activity of CYP11B1, with mitochondrial conversion of deoxycortisol to cortisol (Lechner et al., 2000). Other studies have focused on metyrapone inhibition of corticosterone production (Qiao et al., 2008) or signaling (Vacchio et al., 1994; Pazirandeh et al., 1999), although such observations might be due to inhibition of 11β-HSD1 as shown here, rather than inhibition of CYP11B1. While we did observe CYP11B1 activity in the thymus, this was minimal compared to 11β-HSD1 activity.

The mechanism regulating local glucocorticoid levels confers another means of governing immune development and function. While *de novo* glucocorticoid synthesis from cholesterol is independent of adrenal steroids, glucocorticoid regeneration requires the availability of circulating DHC (or in humans, cortisone). DHC is present in serum of fetal, juvenile, and adult mice (Hundertmark et al., 2002; Tagawa et al., 2007) and in neonatal and adult rats (Tagawa et al., 2007; Obut et al., 2009), suggesting a readily available substrate reservoir throughout development, and one that is energetically efficient. Furthermore, as circulating DHC can increase or decrease in response to different stressors (Obut et al., 2004, 2009), adverse environmental conditions may affect lymphoid glucocorticoid levels via the amount of available substrate. While plasma DHC and cortisone circulate at only 10–30% of plasma corticosterone or cortisol levels (Weber et al., 2000; Kushnir et al., 2004; Obut et al., 2004, 2009), only 5% of plasma corticosterone and cortisol may be available to enter tissues, as 95% is bound to plasma binding proteins (Breuner and Orchinik, 2002; Chapman et al., 2013).

Together, these data suggest that dynamic regulation of DHC or cortisone could be a major mechanism by which adverse environmental conditions regulate lymphocyte development and function, especially under conditions such as chronic stress or disease, which can have long-term programming effects on lymphocytes (Brachman et al., 2015). Furthermore, 11β-HSD1-dependent synthetic glucocorticoids might also preferentially target lymphoid organs and other 11β-HSD1-expressing cells (Sai et al., 2009), and this targeting could contribute to reduced side effects versus 11β-HSD1-independent glucocorticoids such as dexamethasone (Teuffel et al., 2011).

4.3. Age-related change in glucocorticoid production

We compared lymphoid organs of neonatal and young adult mice, and observed a clear decrease in CYP11B1 and increase in 11β-HSD1 with age. This finding is consistent with previous data suggesting reduced glucocorticoid synthesis in thymic epithelial cells (Vacchio et al., 1994) and increased glucocorticoid regeneration in thymocytes (Qiao et al., 2008). Our data additionally show a net increase in lymphoid glucocorticoid production with age.

The role of local glucocorticoid production seems most obvious in early life. Similar to offspring of other altricial species, neonatal mice undergo a stress hyporesponsive period during which adrenal corticosterone production is minimal (Schmidt et al., 2003; Taves et al., 2015). Early life is also when much of the T cell repertoire is generated (Shanley et al., 2009), suggesting that local glucocorticoid production correlates with thymocyte development. We speculate that local glucocorticoid production in the bone marrow and spleen may similarly facilitate B cell development.

The role of increased lymphoid glucocorticoid production in adults, however, is unclear. First, endogenous glucocorticoids contribute to age-related involution of the thymus (Chen et al., 2010), and a steady rise in thymus 11β-HSD1 activity might substantially accelerate this process. Second, while T cell production declines after adolescence, it does continue throughout life, raising the interesting possibility that increased 11β-HSD1 in adults more strongly inhibits TCR signaling, and thus raises the threshold for negative selection. Such a shift could result in survival of T cells with stronger self-recognition, increasing the risk of autoimmunity (Tolosa et al., 1998). Alternatively, such a shift might compensate for age-related defects in thymocyte development (Shanley et al., 2009), instead functioning to maintain competence of naïve T cells. In the bone marrow, increased 11β-HSD1 activity in adults might regulate fate decisions of hematopoietic precursors toward different lineages (Trottier et al., 2008), promote erythrocyte development (Bauer et al., 1999), or as above, shift selection of B cells. Additionally, increased glucocorticoid production could play a role in slowing bone growth in adulthood (Cooper et al., 2002).

4.4. Limitations and future directions

While our experiments clearly show differential activities of CYP11B1 and 11β-HSD1, there are several limitations to these data. First, our enzyme activity studies were performed *in vitro*. Testing the contribution of each enzyme to lymphoid development would require *in vivo* models, such as mouse strains with conditional deletion of each of these enzymes.

Second, we did not examine the availability of endogenous steroid substrates for CYP11B1 and 11β-HSD1, which might limit synthesis or regeneration. While we have measured lymphoid DOC levels (Taves et al., 2015), published data on circulating DHC levels in mice are limited. We are currently developing an LC-MS/MS protocol to measure circulating and tissue DHC levels.

Third, previous data (Vacchio et al., 1994), plus inferences from the age-related changes described here, suggest that CYP11B1 activity might be higher and 11β-HSD1 activity might be lower during prenatal life. We did not examine prenatal timepoints, but future use of fetal thymic organ culture could be a useful tool to explore this possibility. Paired with TCR-transgenic models (Vacchio and Ashwell, 1997; Vacchio et al., 1999), this could also determine CYP11B1 and 11β-HSD1 contributions to lymphocyte selection.

Finally, we have previously found CYP17A1 mRNA and cortisol in mouse lymphoid organs (Taves et al., 2015), and here we detected CYP17A1 mRNA but detected little or no cortisol in fresh or cultured tissues. While the same line of C57BL/6 mice was used in both studies, the mice were housed in different animal facilities. The mice in the present study were re-derived for entry into the facility, with more stringent microbiological exclusion criteria. Thus, while endogenous corticosterone levels were similar in mice from both facilities, the present study did not test the contributions of CYP11B1 and 11β-HSD1 to local cortisol levels. The absence of cortisol in the present study might be due to an unknown environmental difference, such as exposure to different microbiota (Taves, 2015). Future studies will examine this intriguing possibility.

4.5. Conclusions

We demonstrated local glucocorticoid production in lymphoid organs of developing and adult mice, and found that this occurs primarily via corticosterone regeneration from DHC, rather than corticosterone synthesis from DOC. Our findings indicate that glucocorticoid regeneration occurs across lymphoid organs, and increases over development. Together with previous work showing local elevation of endogenous glucocorticoids (Schmidt and Soma, 2008; Taves et al., 2015, 2016), these results indicate that the lymphoid glucocorticoid milieu is strongly regulated by the glucocorticoid-metabolic enzyme 11β-HSD1. This local amplification of glucocorticoids is likely instrumental in selection of developing thymocytes (Mittelstadt et al., 2012), and may be similarly critical in the regulation of mature T cell activation (Zhang et al., 2005) and B cell development (Lechner et al., 2001; Schmidt et al., 2010; Gruver-Yates et al., 2013; Taves et al., 2011a). Local amplification may also be a major contributor to glucocorticoid-induced apoptosis of T and B cells and to thymic involution during chronic stress and aging. However, the use of DHC rather than DOC as a substrate indicates that the local control of lymphoid glucocorticoid concentrations is tied to systemic glucocorticoid metabolites, whose plasma concentrations may be dramatically altered in response to adverse environmental conditions or in disorders that involve dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Targeted inhibition of 11β-HSD enzymes, currently a major goal for the treatment of metabolic syndrome, osteoporosis, and cognitive aging (Gathercole et al., 2013), may also alter immune development and activation.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.05.003.

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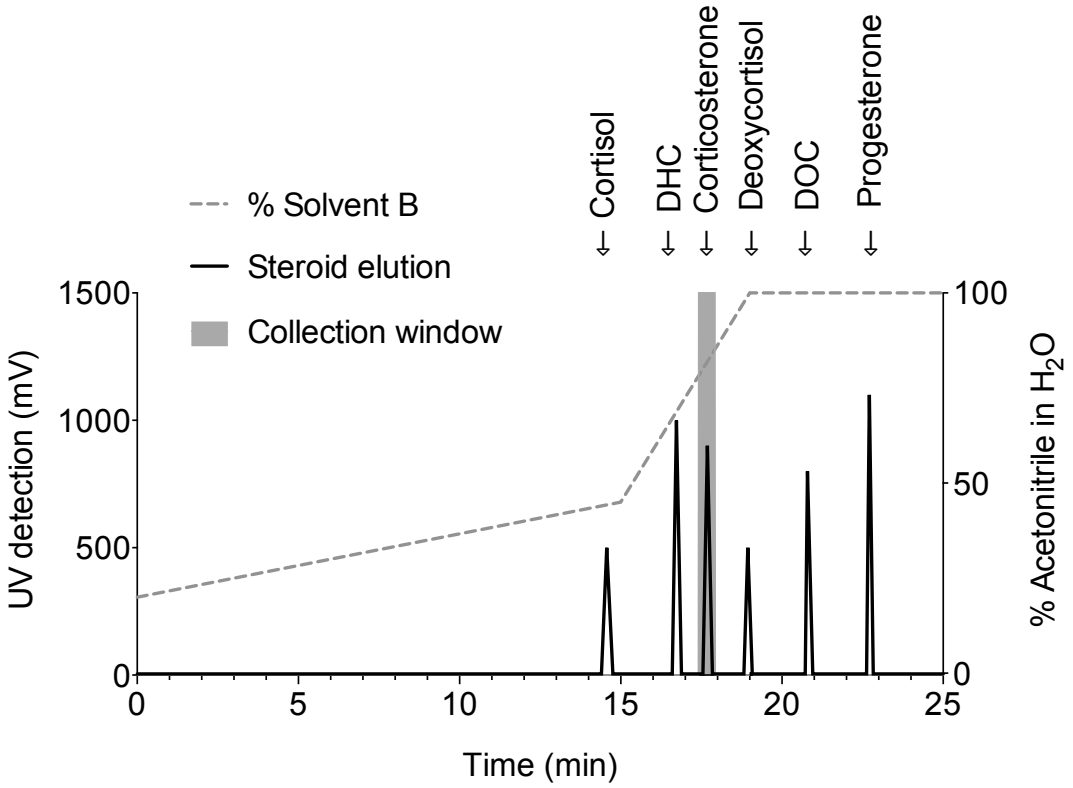
Supplementary Table 1. 5’ nuclease probe qPCR assays

Gene	Forward (5’-3’)	Reverse (5’-3’)	Probe (5’-3’)	Exons
Star	tatctgggtctgcgaatagga	tccctccaagactaaactcact	agccacccctttcaggtcaatactg	6-7
Cyp11a1	gccttaagtcccagtagaagttc	tcgactcctcagaactaagacc	tgaaaatcacatcccaggcagctg	4-5
Hsd3b1	gtctgtccttcccagtgattg	cctgatcttttcagccacca	tttgctctctcagttgtgaccatttcct	1-2
Hsd3b6	cactgtcaccttgatgtttgtatc	gatgcaggagaaagatctggag	tgctccctggtttctggtctgaag	2-3
Cyp21a1	ggttccaggaagcgatctg	caactagggctagcagcatc	tcatcatccccaacatccaaggcg	8-10
Cyp11b1	gccagctcaaagaaagtcat	caggctaactcaatggaactca	actgacacgacatcaacccccttg	5-6
Hsd11b1	atgtccagtccgcccat	gaggaaggtctccagaaggta	ccatagtgccagcaatgtagtgagca	3-5
Hsd11b2	cgtgaagcccatggcat	ggctggatcgcgttgtc	cggttgtgacactggttttggcaa	1-2
Cyp17a1	ccttttccttggtccgacaa	cagagaagtgctcgtgaaga	ctagagtcaccatctggggccga	1-2
Rps29	tccattcaaggtcgcttagtc	cggtctgatccgcaatacg	aagcctatgtccttcgcgtactgc	2-3
Rpl4	cttgccagctctcattctctg	tggtggttgaagataaggttga	ctgaacagcctccttggtcttcttgta	5-6
Oaz1	gccaatgaacgagatcactt	gctgtttaagatggtcaggtga	atttgagccaggggaggtgacac	5-5

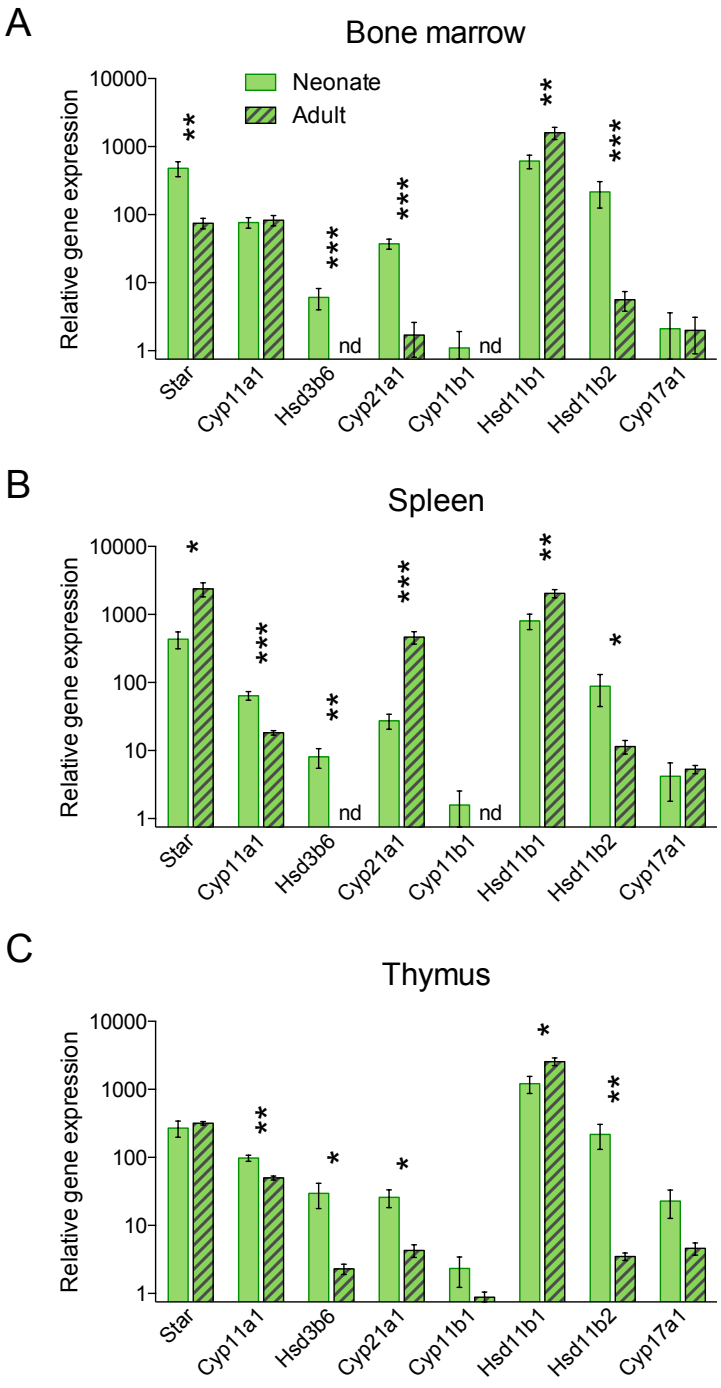
Note: Predesigned 5’ nuclease probe qPCR assays were purchased from Integrated DNA Technologies (cat. # Mm.PT.58.8323852, Mm.PT.58.29648776, Mm.PT.58.43238915, Mm.PT.58.5793064, Mm.PT.58.29960109, Mm.PT.58.41798181, Mm.PT.58.30853261, Mm.PT.58.13323852, Mm.PT.58.10555317, Mm.PT.58.21577577, Mm.PT.58.17609218, Mm.PT.58.33130152.g).

Supplementary Table 2. Monoclonal antibodies used for flow cytometry.

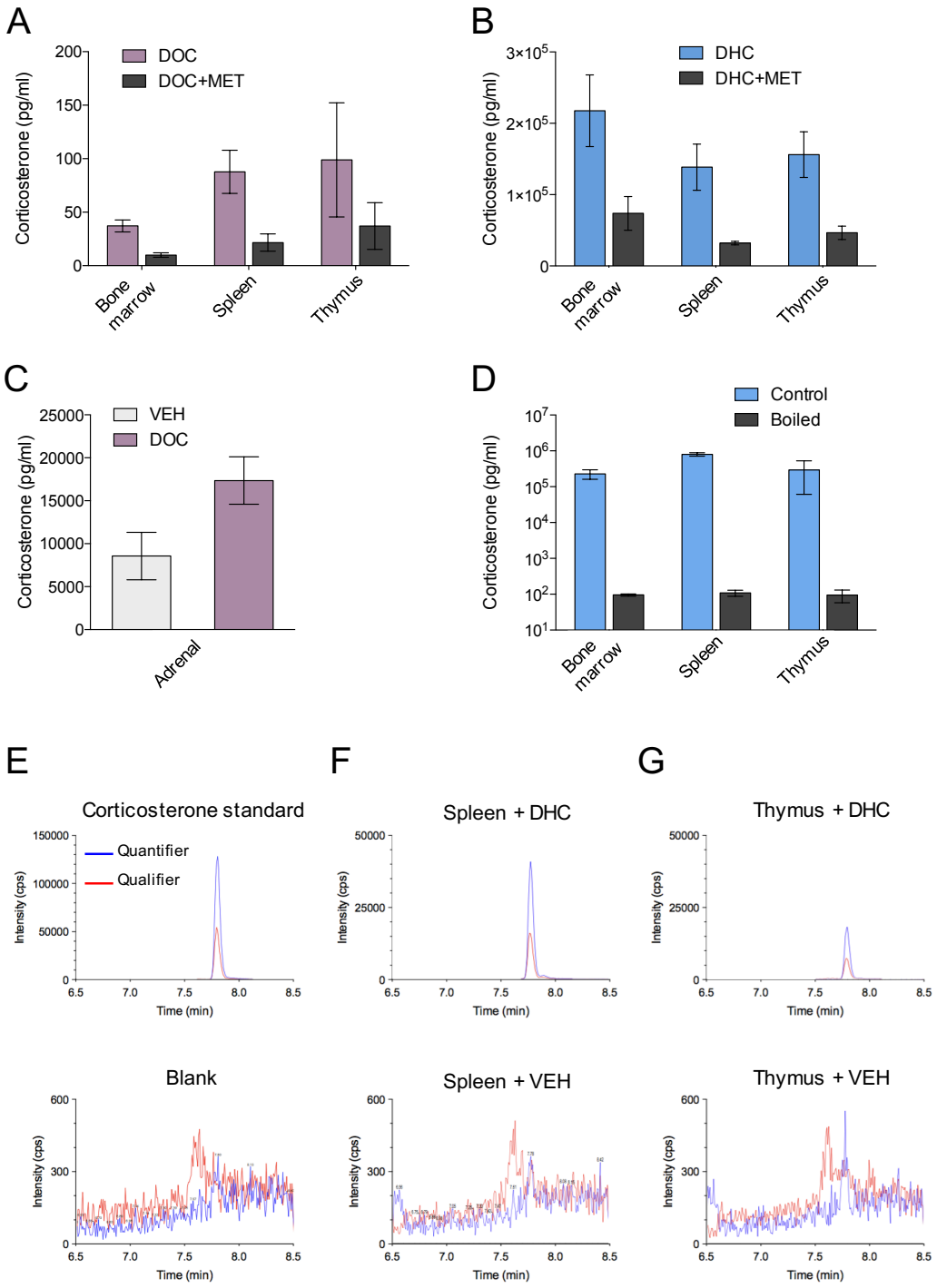
Target	Label	Company	Clone	Catalog #
CD4	Pacific blue	BD Pharmingen	RM4-5	558107
CD4	APC	eBioscience	GK1.5	17-0041-82
CD8α	APC-eFluor 780	eBioscience	53-6.7	47-0081-82
CD45R/B220	PE	BD Pharmingen	RA3-6B2	561878



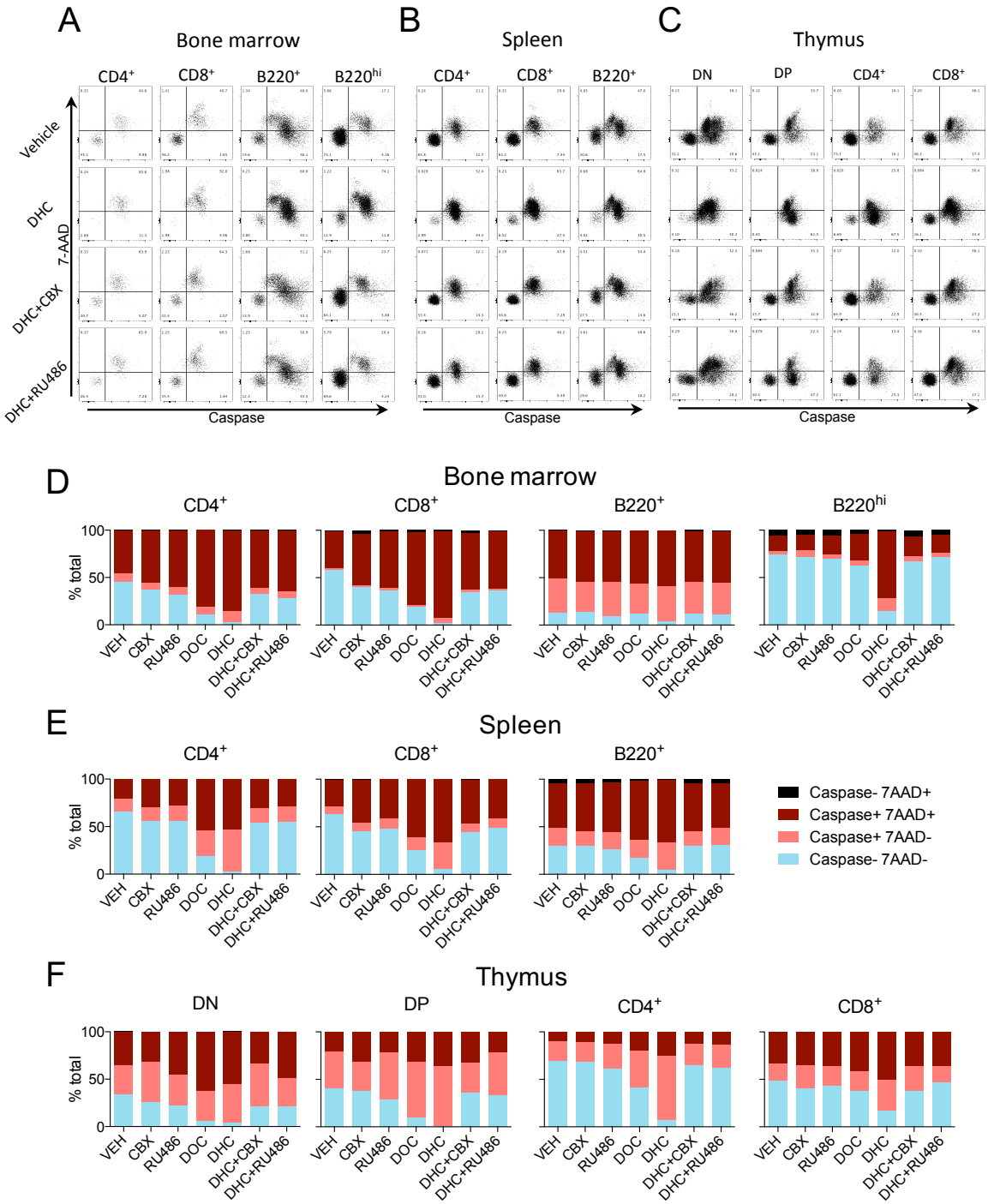
Supplementary Figure 1. HPLC separation of glucocorticoids. Retention times were determined by UV detection (shown above) and corticosterone elution time was confirmed by collection of 3H-labeled corticosterone in 0.1 min fractions and counting in a liquid scintillation counter (data not shown). DHC = dehydrocorticosterone, DOC = deoxycorticosterone.



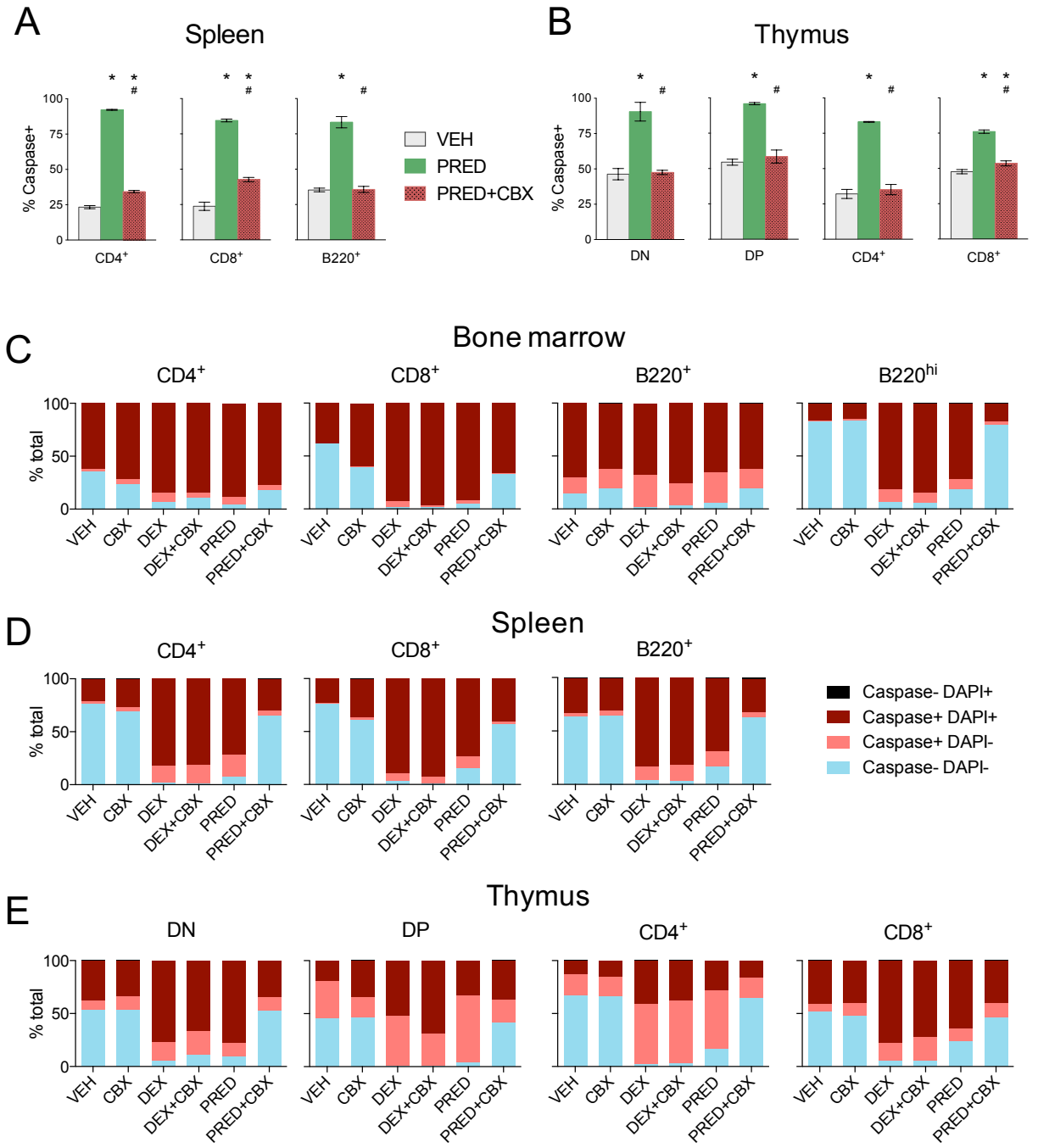
Supplementary Figure 2. Relative gene expression of steroidogenic enzymes in neonatal and adult (**A**) bone marrow, (**B**) spleen, and (**C**) thymus, as determined using highly specific 5' nuclease probe-based qPCR assays. Expression was corrected using the mean of three reference genes *Rps29*, *Rpl4*, and *Oaz1*, and expressed relative to adult thymus *Cyp11b1* (note the log scale for the y-axes). In some cases, gene expression was not detectable (nd). *Hsd3b1* expression was not detectable in any age or tissue. N = 9 neonates, 8 adults.



Supplementary Figure 3. Corticosterone production by adult tissues in the presence of (**A**) 1 μM DOC, or (**B**) 1 μM DHC was inhibited by co-incubation with 0.9 mM metyrapone (MET). (**C**) Adrenal corticosterone production was increased in the presence of DOC. (**D**) DHC conversion to corticosterone was abolished by boiling tissue samples before incubation. All N = 3. (**E-G**) Representative LC-MS/MS multiple reaction monitoring (MRM) traces confirming RIA corticosterone measurements. (**E**) Corticosterone standard or blank, (**F**) samples from DHC- and vehicle-incubated spleen, and (**G**) samples from DHC- and vehicle-incubated thymus.



Supplementary Figure 4. DHC regeneration induces apoptosis of T and B lymphocytes. (**A-C**) Representative FACS plots and (**D-F**) lymphocyte subsets staining for Caspase 3/7 activity (induction of apoptosis) and 7-AAD (loss of membrane integrity). Total bone marrow cells, splenocytes, and thymocytes were cultured with vehicle (VEH), 0.1 μM deoxycorticosterone (DOC), 0.1 μM dehydrocorticosterone (DHC), 1 mM carbenoxolone (CBX), or 1 μM RU-486. N = 3 per experiment, one of two experiments shown. DN = $CD4^{-}CD8^{-}$ double negative thymocytes, DP = $CD4^{+}CD8^{+}$ double positive thymocytes.



Supplementary Figure 5. Prednisone regeneration induces apoptosis of T and B lymphocytes. (**A-B**) Caspase 3/7 activity and (**C-E**) lymphocyte subsets staining for Caspase 3/7 activity (induction of apoptosis) and DAPI (loss of membrane integrity). Total bone marrow cells, splenocytes, and thymocytes were cultured with vehicle (VEH), 0.1 μM prednisone (PRED), 0.1 μM dexamethasone (DEX), or 1 mM carbenoxolone (CBX). N = 3 per experiment, one of two experiments shown. DN = $CD4^{-}CD8^{-}$ double negative thymocytes, DP = $CD4^{+}CD8^{+}$ double positive thymocytes.