Published in final edited form as:
J Immunol. 2018 March 15; 200(6): 1988–1994. doi:10.4049/jimmunol.1701328.

De novo glucocorticoid synthesis by thymic epithelial cells regulates antigen-specific thymocyte selection

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Abstract

Glucocorticoid (GC) signaling in thymocytes counters negative selection and promotes the generation of a self-tolerant yet antigen-responsive T-cell repertoire. Whereas circulating GC are derived from the adrenals, GC are also synthesized *de novo* in the thymus. The significance of this local production is unknown. Here we deleted 11β-hydroxylase (Cyp11b1), the enzyme that catalyzes the last step of GC biosynthesis, in thymic epithelial cells (TEC) or thymocytes. Like glucocorticoid receptor (GR)-deficient T cells, T cells from mice lacking TEC-derived but not thymocyte-derived GC proliferated poorly to alloantigen, had a reduced anti-viral response, and exhibited enhanced negative selection. Strikingly, basal expression of GC-responsive genes in thymocytes from mice lacking TEC-derived GC was reduced to the same degree as in GR-deficient thymocytes, indicating that at steady state the majority of biologically-active GC are paracrine in origin. These findings demonstrate the importance of extra-adrenal GC even in the presence of circulating adrenal-derived GC.

Introduction

Generation of a competent but self-tolerant T cell antigen-specific repertoire takes place in the thymus. The fate of $CD4^+CD8^+$ (double positive, or DP) thymocytes is determined by recognition of self peptides presented by MHC molecules (self-pMHC). DP cells with TCRs that do not recognize self-pMHC presented by cortical thymic epithelial cells (cTEC) die “by neglect”. Those that recognize self-pMHC enter the medulla where they encounter migratory dendritic cells (DC), some of which present self-pMHC derived from peripheral tissues, medullary TEC (mTEC) in which the autoimmune regulator (Aire) drives expression of tissue-restricted antigens, and resident DC bearing peptides transferred from mTEC (1). DP cells having TCRs with strong avidity for self-pMHC die (negative selection) whereas those with intermediate avidity survive (positive selection) and populate the periphery (2, 3).

Glucocorticoids (GC) are steroid hormones that bind the glucocorticoid receptor (GR), a ligand-dependent transcription factor that translocates to the nucleus and regulates transcription by binding to its response elements or other transcription factors. GC potently downregulate the production of pro-inflammatory cytokines, chemokines, and

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prostaglandins, and antagonize NF-κB and AP-1 (4). GC also inhibit transcriptional activity of Nur77 (5), a TCR-induced transcription factor implicated in thymocyte negative selection (6, 7). We previously suggested that by blunting TCR signals at a distal step (i.e. in the nucleus), GC could raise the threshold of avidity for self-pMHC above which negative selection takes place, allowing positive selection of TCRs that would otherwise be negatively selected (8). Evidence for an effect of GC on thymocyte selection was initially obtained from fetal thymic organ cultures in which negative selection of TCR-transgenic thymocytes was increased by pharmacologic inhibition of local GC production (9). This was subsequently supported by *in vivo* studies in which GR expression was reduced by the expression of an antisense transgene (10-12). The best evidence has been obtained with mice in which the GR was deleted in thymocytes (13). T cells from these mice responded normally to repertoire-independent TCR stimuli, but had diminished responses to immunization with foreign antigen, infection with lymphocytic choriomeningitis virus (LCMV) Armstrong strain, and culture with allogeneic APC, indicating a decrease in the avidity with which the repertoire recognized pMHC (13). Alterations of the TCR repertoire were confirmed by analysis of TCR Vβ CDR3 sequences.

Although circulating GC are primarily produced in the adrenal cortex, the thymus is itself a site of synthesis (14-19). Cultured mouse and chicken TECs express GC-synthetic enzymes and secrete steroid intermediates and GC themselves, production being highest at birth when adrenal production of GC is lowest (14, 17, 20). Direct measurement of thymus GC found corticosterone and its precursor steroid concentrations to be higher than in blood, particularly shortly after birth, confirming thymic GC synthesis *in vivo* (19). In addition to TEC, it has been proposed that thymocytes themselves are a source of GC, especially later in life (18). The functional contribution of extra-adrenal GC synthesis in the thymus, or any tissue for that matter, is unknown. To address this, we conditionally deleted Cyp11b1 (P450 c11b1), the enzyme that catalyzes the conversion of biologically inactive precursors to active GC, in TEC or thymocytes, and characterized the results in thymocytes and T cells.

Materials and Methods

Mice

C57BL/6 (B6) and the congenic strains *B10.A* and *Rag2*$^{-/-}$, AND TCR-transgenic mice (21), *β-actin-FLPe* (22), *FoxN1-Cre*-transgenic mice (23), and *β-actin-Cre* mice were obtained from Jackson Laboratory. *Lck-Cre*–transgenic mice were obtained from Taconic. *Nr3c1* (GR) exon 3 conditionally targeted mice were described (13). A conditional *Cyp11b1* allele with *loxp* sites flanking exons 3-5 was generated by recombineering (24) (Supplemental Fig. 1A) and *Cyp11b1*-floxed mice were generated using C57BL/6 ES cells. The Neo cassette was removed by crossing floxed mice with *β-actin-FLPe* transgenic mice. All mice used in this study were backcrossed for at least 6 generations onto B6. Primer sequences used for genotyping are provided in Supplemental Table 1.

Antibodies

Anti-CD3 (145-2C11) and anti-CD28 (37.51) were from BD Pharmingen. For flow cytometry, antibodies recognizing CD45.2 (104), CD4 (RM4-5), and PD-1 (J43) were from eBioScience, recognizing Helios (22F6) from BioLegend, and recognizing Bim (C34C5) from Cell Signaling Technology. Antibodies against EpCAM (G8.8), MHC-II (M5/114.15.2), CD8α (53-6.7), and TCRβ (H57), as well as Annexin V, were from BD Pharmingen.

Measurement of corticosterone

Corticosterone was measured by chemiluminescence ELISA (Arbor Assays).

Cell culture and T cell proliferation

T cells were cultured in RPMI 1640 (Biofluids) supplemented with 10% heat-inactivated calf serum (Sigma), 100 mg/ml gentamicin, 4 mM glutamine, and 50 μM 2-mercaptoethanol. To measure T cell proliferation, 3×10^4 (anti-CD3/CD28) or 1.5×10^5 (alloantigen) purified lymph node T cells were cultured in triplicate in a total volume of 200 μl complete medium with 0.5 μg/ml plate-bound anti-CD28 and the indicated amounts of plate-bound anti-CD3, or with the indicated numbers of irradiated B10.A splenocytes in 96-well plates. After 48 (anti-CD3/anti-CD28) or 72 (alloantigen) hr wells were pulsed overnight with [^{3}H]-thymidine and harvested. Incorporation of radioactivity was determined using a 1450 Microbeta liquid scintillation counter (Wallac).

Cell purification and flow sorting

Thymic epithelial cells were isolated by digestion of minced thymi and enrichment with discontinuous percoll as described (25). Genomic DNA from sorted TECs (Epcam$^+$, MHC-II$^+$, and CD45.2$^-$) and DP thymocytes (TCRβ$^+$, CD4$^+$CD8α$^+$) was purified using a DNeasy kit (Qiagen). Sorts were performed with a FACSAria II or a FACSAria Fusion (BD Bioscience). T cells used in proliferation assays were purified from lymph nodes using Dynabead Untouched Mouse T cell kits (Invitrogen).

RT-PCR

Total RNA was isolated with an RNeasy Mini kit (Qiagen) and cDNA generated with Superscript RT (Invitrogen). Real-time PCR was performed with SYBR Green PCR mix (Applied Biosystems) using a QuantStudio 6 (Applied Biosystems). The results are relative to *HPRT* expression. Primer sequences used for real-time PCR are provided in Supplemental Table 1.

Viral infection

LCMV Armstrong was obtained from Dorian McGavern (NINDS). Mice were inoculated i.p. with 2×10^5 PFU. Splenocytes were stained with APC-labeled class I tetramers containing LCMV peptides gp33, gp276, and np396 (NCI tetramer core facility).

LPS treatment in vivo

LPS from *E. coli* (Sigma #L2880) was solubilized in PBS and injected i.p. at a dose of 3 μg/gram body weight. Control mice were injected with PBS alone.

Statistical analysis

Unless otherwise indicated, statistical analyses were performed using GraphPad Prism software and an unpaired 2-tailed Student's *t* test. *P* values 0.05 or less were considered significant. Averaged results of multiple experiments are presented as the arithmetic mean ± SEM.

Results

Generation and functional characterization of a conditional Cyp11b1 allele

Mice with global deletion of exons 3-7 of *CYP11B1* have markedly diminished adrenal corticosterone production (26). To address the role of thymus-derived GC in thymocyte development, we generated mice in which *CYP11B1* could be disrupted in a tissue-specific manner. Mice were generated in which LoxP sites flanked *CYP11B1* exons 3-5 ($Cyp11b1^{\mathrm{fl/fl}}$ mice, Supplemental Fig. 1A). Deletion of these exons results in early termination of translation after an open reading frame containing the first 135 of 501 amino acids followed by 5 out-of-frame residues (Supplemental Fig. 1B). These mice were crossed with animals expressing *actin*-Cre to delete *CYP11B1* in the entire germline ($Cyp11b1^{-/-}$ mice). Cultured wild-type but not $Cyp11b1^{-/-}$ adrenal cells synthesized substantial amounts of corticosterone, which was prevented by the Cyp11b1 inhibitor metyrapone (Fig. 1A). Plasma corticosterone levels in $Cyp11b1^{-/-}$ mice were < 50% of WT levels, similar to the reduction reported for *Cyp11b1* exon 3-7-targeted mice (26), but were not statistically significantly different from levels in $Cyp11b1^{foxN1\text{-}Cre}$ animals (Fig. 1B). Systemic GC increase in response to an acute stress such as LPS (27). Intraperitoneal injection of LPS increased plasma corticosterone levels in WT but not $Cyp11b1^{-/-}$ mice (Fig. 1C). Finally, $Cyp11b1^{-/-}$ mice exhibited adrenal hyperplasia (Fig. 1D), characteristic of impaired glucocorticoid production (26).

TEC, not thymocytes, are the major source of thymic GC

Cyp11b1 was deleted in TEC or thymocytes by crossing $Cyp11b1^{\mathrm{fl/fl}}$ mice with mice expressing Cre knocked into the *FoxN1* locus (23) ($Cyp11b1^{foxN1\text{-}Cre}$ mice) or expressed as a transgene driven by the proximal *lck* promoter ($Cyp11b1^{lck\text{-}Cre}$ mice) (28). The tissue specificity of deletion was shown by PCR of genomic DNA from sort-purified cells, which demonstrated that *Cyp11b1* exon 4 was absent in $Cyp11b1^{foxN1\text{-}Cre}$ TEC but not DP thymocytes, with the opposite being true for $Cyp11b1^{lck\text{-}Cre}$ cells (Fig. 2A). Because stress-induced elevations in adrenal-derived GC can cause acute thymic involution (29) it is assumed that the thymus also responds to circulating levels at steady-state. However, the relative contributions of systemic *versus* paracrine GC have never been experimentally addressed. To do this, the constitutive expression of two GC-responsive genes, *Gilz* and *Lad1* (30) was used as a read-out of GC signaling in freshly-isolated thymocytes. In GR-deficient thymocytes ($GR^{lck\text{-}Cre}$), *Gilz* and *Lad1* mRNA levels were reduced 40-50% compared to WT controls (Fig. 2B). *Gilz* and *Lad1* expression were similarly reduced in $Cyp11b1^{-/-}$ thymocytes, as expected, and also in $Cyp11b1^{foxN1\text{-}Cre}$ thymocytes, in which only TEC-synthesized GC are absent. In contrast, this reduction was not observed in thymocytes of $Cyp11b1^{lck\text{-}Cre}$ mice, or in *Cyp11b1* heterozygous ($Cyp11b1^{fl/+,actin\text{-}Cre}$) thymocytes. Thymocyte expression of the GC-unresponsive gene *Calm3* was similar across genotypes. Differences in *Gilz* and *Lad1* expression were not due to GC hyporesponsiveness, as they were induced by exogenous GC in all GR-sufficient thymocytes (Supplemental Fig. 1C). Together, these data show that biologically-active thymus GC are synthesized *de novo* by TEC, not thymocytes, *in situ*. Furthermore, under basal conditions, thymocyte GC signaling is primarily driven by TEC- rather than adrenal-derived GC.

Negative selection is enhanced in the absence of TEC-derived GC

Deletion of the GR in immature thymocytes results in the negative selection of cells that otherwise would have been positively selected (13). One example was the reduction in DP and CD4$^+$ thymocytes in AND mice whose transgenic TCR normally promotes positive selection of CD4$^+$ T cells in H-2^b animals. To prevent rearrangement of endogenous TCRα, recombinase-activating gene 2 (*RAG*-2) was deleted by crossing AND with *RAG2*$^{-/-}$ mice. As observed with AND *GR*$^{lck\text{-}Cre}$ mice whose thymocytes cannot respond to GC (13), there was a reduction in the number of DP and CD4$^+$ SP thymocytes in *Cyp11b1*$^{foxN1\text{-}Cre}$ AND mice compared to *Cyp11b1*$^{fl/fl}$ AND controls (Fig. 3A). Among the molecules upregulated during negative selection are PD-1, Helios, and the pro-apoptotic Bcl-2 family member Bim (31). Furthermore, Annexin V binds to cells actively undergoing apoptosis (32). We examined pre-selection thymocytes (DP), TCR-signaled DP thymocytes (TCR^{hi}, (33)), a population of cells enriched for those undergoing negative selection (CD4^{low}/CD8^{low} “double dull” (34-36)), and mature thymoytes (CD4$^+$ SP). We found that double dull, and to a lesser extent SP, thymocytes from *Cyp11b1*$^{foxN1\text{-}Cre}$ AND mice had a larger fraction of Bim^{hi}PD-1$^+$ cells compared to *Cyp11b1*$^{fl/fl}$ AND controls (Fig. 3B). Strikingly, the fraction of apoptotic cells (Helios^{hi}Annexin V$^+$) was increased in *Cyp11b1*$^{foxN1\text{-}Cre}$ thymocytes, most notably in the double dull subset. Therefore, loss of TEC-derived GC mice resulted in increased negative selection of thymocytes that normally undergo positive selection.

Cyp11b1$^{foxN1\text{-}Cre}$ T cells have decreased response to allo- and foreign antigens

A hallmark of the changed repertoire in GR-deficient T cells is a decreased allogenic response (13). If paracrine production by TEC is the major source of intrathymic GC, *Cyp11b1*$^{foxN1\text{-}Cre}$ T cells would also be expected to have a reduced response to allogeneic APC. To test this, H-2^b T cells were cultured with irradiated H-2^a splenocytes. Whereas *Cyp11b1*$^{foxN1\text{-}Cre}$ T cells responded normally to stimulation with plate-bound anti-CD3/anti-CD28, the response of *Cyp11b1*$^{foxN1\text{-}Cre}$ T cells to allogeneic stimulation was blunted (Fig. 4A). A possible contribution of thymocyte-synthesized GC on the TCR repertoire was addressed by parallel experiments using *Cyp11b1*$^{1ck\text{-}Cre}$ T cells as responders. The response of WT and *Cyp11b1*$^{1ck\text{-}Cre}$ T cells was identical to both anti-CD3/anti-CD28 and allogeneic APC (Fig. 4B).

Similar to alloantigen, the altered TCR repertoire in *GR*$^{lck\text{-}Cre}$ caused T cells to respond less well when mice were infected with LCMV Armstrong strain (13), which elicits a robust CD8$^+$ T cell response that peaks at seven days. WT and *Cyp11b1*$^{foxN1\text{-}Cre}$ mice were infected with the LCMV and seven days later splenic T cells were characterized. *Cyp11b1*$^{foxN1\text{-}Cre}$ mice had a 15% and 30% reduction in CD4$^+$ and CD8$^+$ T cell subsets, respectively, compared to WT (Fig. 5A). There was a decrease in the number of LCMV gp33-specific CD8$^+$ T cells in spleens of *Cyp11b1*$^{foxN1\text{-}Cre}$ mice as measured by peptide-MHC I tetramer binding. There was also a decrease in LCMV np396-reactive CD8 T cells, although the reduction did not achieve statistical significance. In contrast, targeting Cyp11b1 expression in thymocytes did not affect the response to LCMV (Fig. 5B). Thus, T cells that developed in the absence of TEC- but not thymocyte-provided GC have a reduced ability to respond to pMHC.

Discussion

It was long assumed that GC acting on the thymus were derived from the circulation. However, the discovery that TEC can produce GC (14, 16) raised the possibility that the locally-derived product was biologically active. In fact, blockade of glucocorticoid production in fetal thymic organ culture resulted in increased TCR-mediated activation and enhanced negative selection (15). To assess the relative contribution of local versus systemic GC in the thymus, we quantified the expression of GC-responsive genes in thymocytes at steady-state. Remarkably, the lack of local synthesis reduced expression of these genes to the same levels as in GR-deficient thymocytes, which cannot respond to GC at all. This implies that the bulk of biologically active GC in the thymus under basal conditions are supplied by TEC in a paracrine manner. This discrepancy with the classical understanding of endocrine glucocorticoid signaling may be explained by enhanced bioavailability. Interaction between thymocytes and TECs is a prerequisite for the TCR-pMHC interactions underlying selection. Paracrine delivery via cell-cell contact raises the possibility that GC pass directly from TECs to thymocytes without diffusion and dilution into the extracellular space. In addition, hormone delivered directly to thymocytes would bypass carrier proteins. Approximately 80-90% of plasma GC are bound by the corticosteroid-binding globulin (CBG) and another 5-10% by albumin, leaving only 5% free and available to signal by entering the cell and binding the GR (37). GC that pass directly from TEC to thymocytes would therefore have a much higher effective concentration than those delivered via the blood.

It has been reported that thymocytes express the complete set of steroidogenic enzymes and can produce measurable corticosterone in vitro (18, 38, 39). Thymocytes from adult (14-22 weeks of age) mice produced more GC than those from younger mice, leading to the suggestion that thymocytes supplant TECs for GC production later in life (18). We and others were unable to detect thymocyte Cyp11b1 activity *in vitro* (data not shown and 40). We were, however, able test for a role for thymocyte-produced GC genetically by deleting Cyp11b1 in thymocytes and T cells. Expression of GC-sensitive genes in *Cyp11b1*$^{lck\text{-}Cre}$ thymocytes was normal, indicating that GC levels sensed by thymocytes at the population level were normal. As indicated by the response to allogeneic APC and LCMV, the repertoire of *Cyp11b1*$^{lck\text{-}Cre}$ T cells was also normal.

The data in this report demonstrate a paracrine mode of action by GC produced in the thymus, where effects of GC have previously been ascribed to hormonal control via adrenal production. Awareness of the impact of locally-produced GC could aid development of targeted therapies addressing thymocyte development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Bei Dong for expert technical assistance and Dorian McGavern for the gift of LCMV.

This work was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

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Abbreviations

GC	glucocorticoids
Cyp	cytochrome P450
Cyp11b1	11β-hydroxylase
GR	glucocorticoid receptor
TEC	thymic epithelial cells
DP	double positive thymocytes

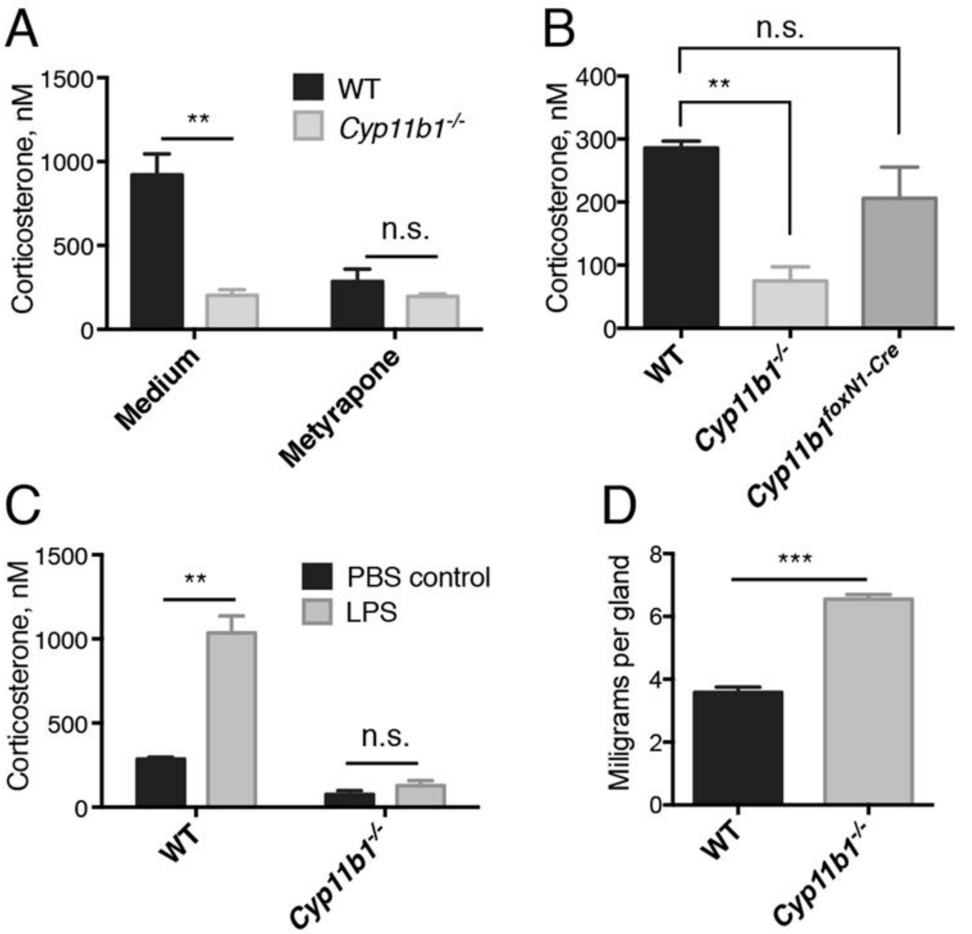

FIGURE 1. Lack of corticosterone production in *Cyp11b1* exon 3-5$^{-/-}$ (*Cyp11b1*$^{-/-}$ mice) mice

(**A**) Corticosterone concentrations in the supernatants of adrenals from WT and *Cyp11b1*$^{-/-}$ mice cultured for three days in the absence or presence of metyrapone. Adrenals from each mouse were cultured in the absence or presence of 200 μg/ml metyrapone. (**B**) Corticosterone levels in plasma from WT, *Cyp11b1*$^{foxN1\text{-}Cre}$, and *Cyp11b1*$^{-/-}$ mice. (**C**) Corticosterone levels in plasma from WT and *Cyp11b1*$^{-/-}$ mice 3 hr after injection LPS or PBS alone. (**D**) *Cyp11b1*$^{-/-}$ mice exhibit adrenal hyperplasia. Weights of adrenals from WT and *Cyp11b1*$^{-/-}$ mice. All data in this figure are shown as the mean ± SEM with n=3. *$P < 0.05$, **$P < 0.005$.

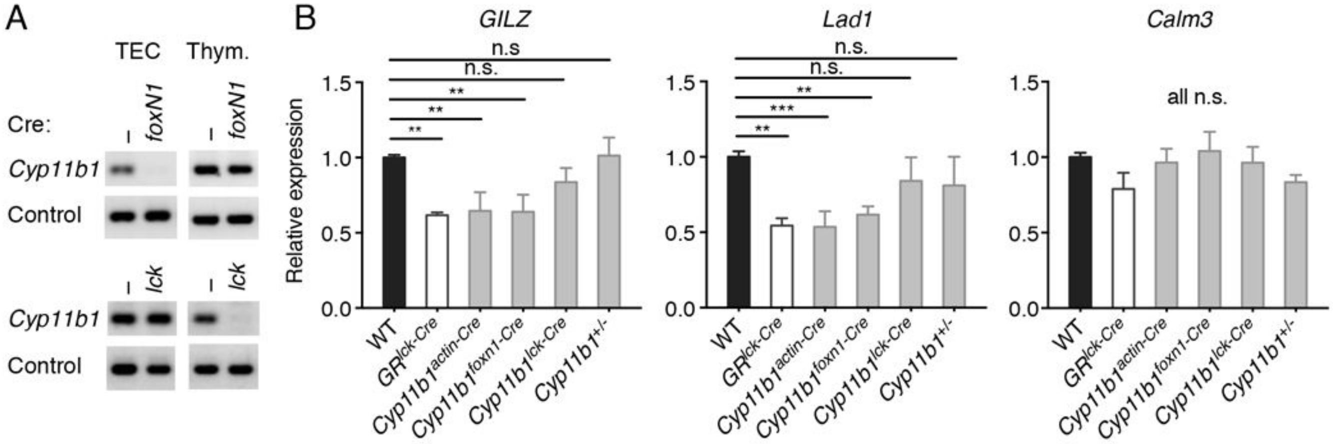

FIGURE 2. Reduced glucocorticoid-dependent gene expression in *Cyp11b1*$^{foxN1\text{-}Cre}$ thymocytes

(**A**) Cre-mediated disruption of *Cyp11b1*. Genomic DNA from sorted TECs and DP thymocytes from WT and *Cyp11b1*$^{foxN1\text{-}Cre}$ (*N1-Cre*) and *Cyp11b1*$^{lck\text{-}Cre}$ (*lck-Cre*) mice was analyzed by PCR for the presence of *CYP11B1* exon 4. Control primers were specific for the H-2A locus (41). One representative pair of three sets of mice for each Cre is shown. (**B**) mRNA levels of GC-sensitive and -insensitive genes in *Cyp11b1*$^{foxN1\text{-}Cre}$ thymocytes. Relative mRNA levels in thymocytes either freshly isolated or after 3 hr of treatment with 100 nM corticosterone were determined by RT-PCR. Significance was determined by 1-way ANOVA, followed by Fisher's LSD multiple comparison (each mutant vs control, n =4 to 8). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

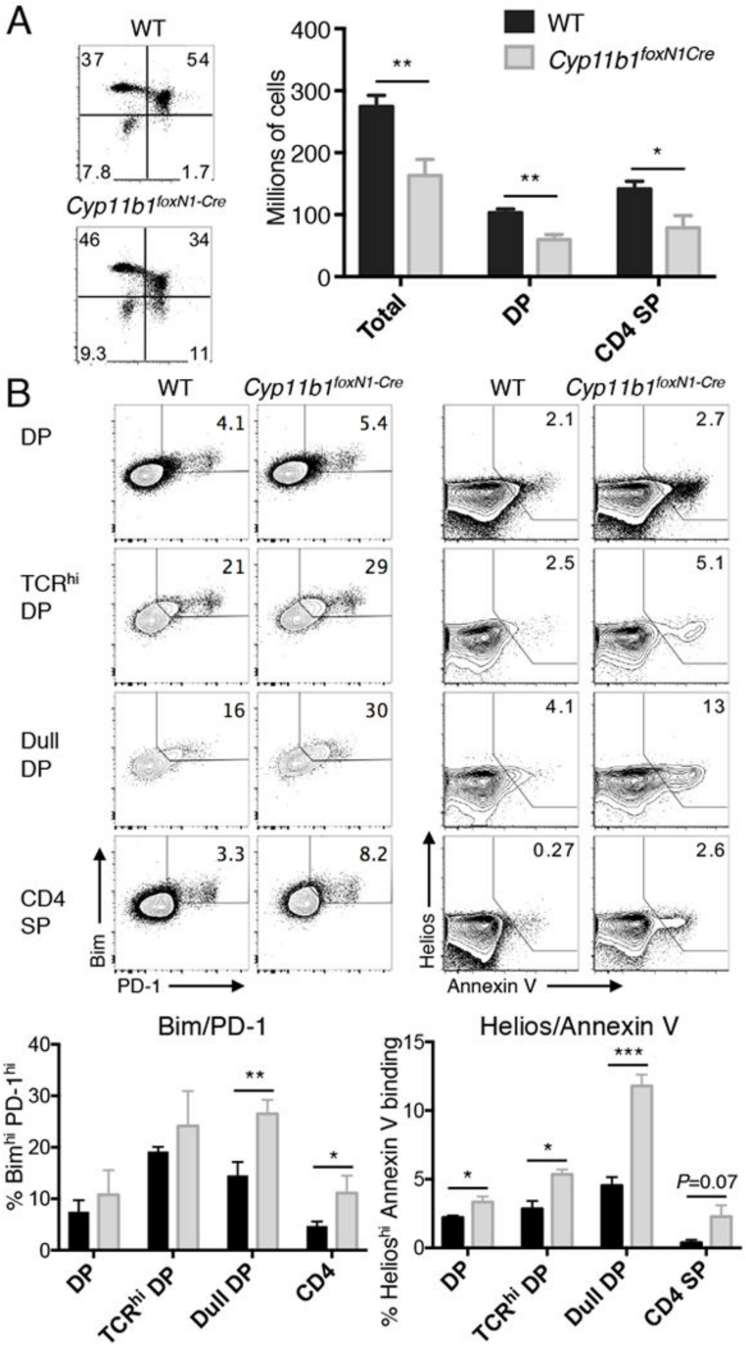

FIGURE 3. Increased negative selection of thymocytes developing in the absence of corticosterone production by TECs

(**A, left panels**) CD4 versus CD8 profiles of representative 5 week-old *AND TCR* WT and *Cyp11b1*$^{foxN1\text{-}Cre}$ thymi. The numbers represent the percentages in each quadrant. (**A, right panel**) Total thymocytes and subsets from five week-old WT (n=10) and *Cyp11b1*$^{foxN1\text{-}Cre}$ (n=6) *Rag2*$^{-/-}$ mice. (**B**) Increased indicators of negative selection in *Cyp11b1*$^{foxN1\text{-}Cre}$ *AND TCR* thymocytes. PD-1 and Bim expression (**upper left panels**) and Helios expression and Annexin V-binding (**upper right panels**) are shown in the indicated subsets of WT and

Cyp11b1$^{foxN1\text{-}Cre}$ *AND TCR* thymocytes. Shown below each are the means and SEM of three (Bim/PD-1) and four (Helios/Annexin V) mice. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

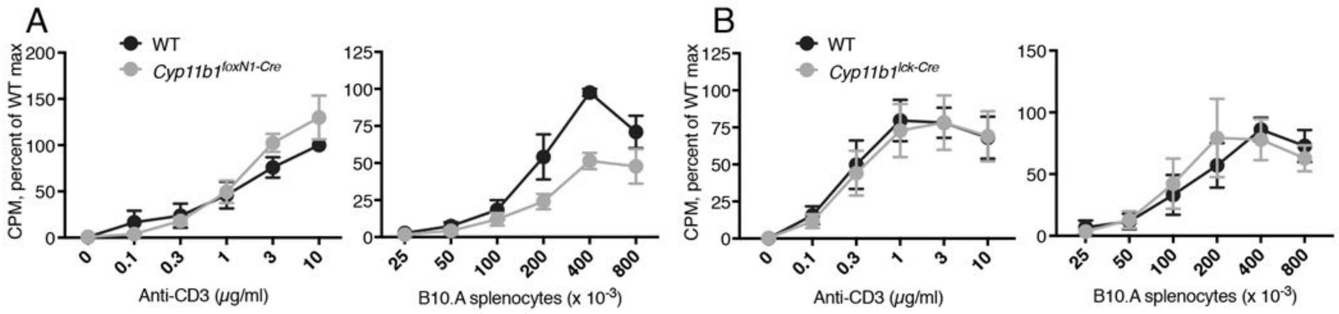

FIGURE 4. The repertoires of *Cyp11b1*$^{foxN1\text{-}Cre}$ T cells, but not of *Cyp11b1*$^{lck\text{-}Cre}$ T cells, were weakened

Cyp11b1$^{foxN1\text{-}Cre}$ T cells proliferate normally (**A**) to cross-linked CD3 (anti-CD3/CD28) but not (**B**) to alloantigen. *Cyp11b1*$^{lck\text{-}Cre}$ T cells proliferate normally (**C**, **D**) to TCR cross-linking and alloantigen. Data are presented as the averaged percent of WT maximum from four (*Cyp11b1*$^{foxN1\text{-}Cre}$) and four (*Cyp11b1*$^{lck\text{-}Cre}$) independent experiments. Data are shown as mean ± SEM.

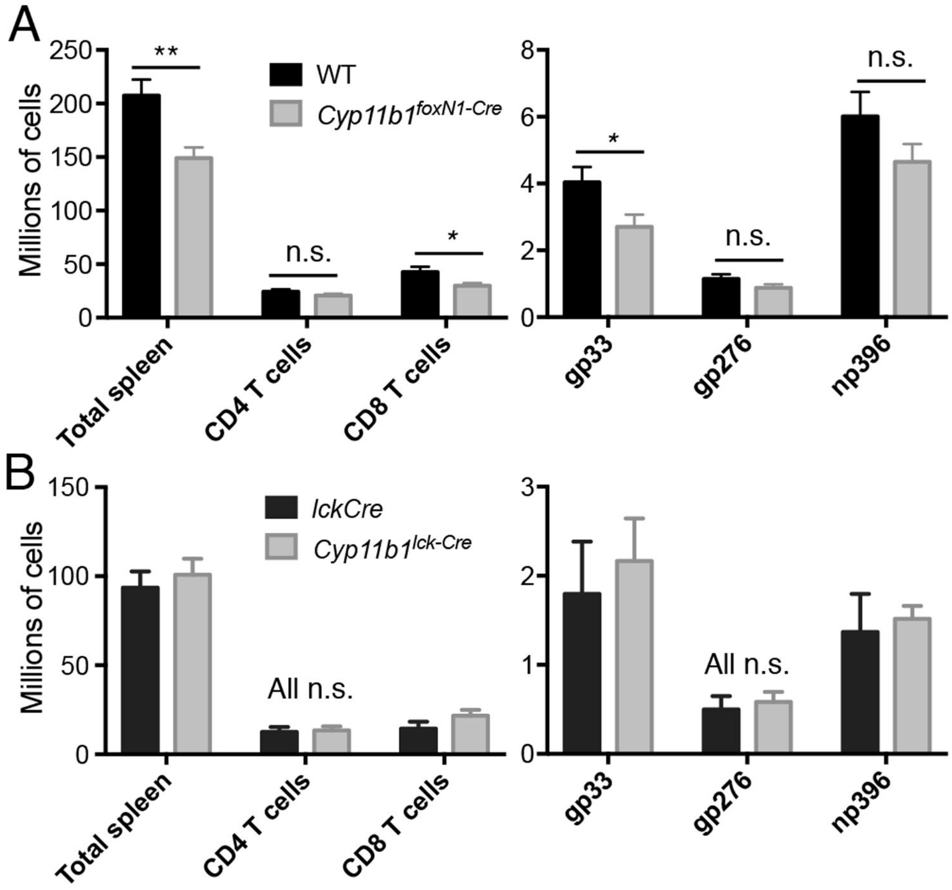

FIGURE 5. Reduced CD8$^+$ T cell response in LCMV-infected *Cyp11b1$^{foxN1\text{-}Cre}$* but not *Cyp11b1$^{lck\text{-}Cre}$* mice

Mice were infected with LCMV Armstrong and splenocytes were analyzed 7 days later. Shown are (left) numbers of splenocytes and T cells and (right) numbers of MHC class I tetramer$^+$ CD8$^+$ T cells from mice of the indicated genotypes (WT, n=12, *Cy11b1$^{foxN1\text{-}Cre}$*, n=11; *lckCre* alone, n=6, *Cyp11b1$^{lck\text{-}Cre}$*, n=6). Data represent the mean ± SEM. *$P < 0.05$, **$P < 0.005$.