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Research Article **Immunology**

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Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness

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Generation of a self-tolerant but antigen-responsive T cell repertoire occurs in the thymus. Although glucocorticoids are usually considered immunosuppressive, there is also evidence that they play a positive role in thymocyte selection. To address the question of how endogenous glucocorticoids might influence the adaptive immune response, we generated $GR^{lck\text{-}Cre}$ mice, in which the glucocorticoid receptor gene (*GR*) is deleted in thymocytes prior to selection. These mice were immunocompromised, with reduced polyclonal T cell proliferative responses to alloantigen, defined peptide antigens, and viral infection. This was not due to an intrinsic proliferation defect, because GR-deficient T cells responded normally when the TCR was cross-linked with antibodies or when the T cell repertoire was "fixed" with αβ TCR transgenes. Varying the affinity of self ligands in αβ TCR transgenic mice showed that affinities that would normally lead to thymocyte-positive selection caused negative selection, and alterations in the TCR repertoire of polyclonal T cells were confirmed by analysis of TCR Vβ CDR3 regions. Thus, endogenous glucocorticoids are required for a robust adaptive immune response because of their promotion of the selection of T cells that have sufficient affinity for self, and the absence of thymocyte glucocorticoid signaling results in an immunocompromised state.

Introduction

Selective processes that give rise to a functional and largely self-tolerant T cell repertoire take place in the thymus and require occupancy of the T cell receptor for antigen (TCR) by peptide-loaded MHC-encoded molecules (self pMHC). The majority of $CD4^+CD8^+$ (double-positive [DP]) thymocytes fail to express TCRs of sufficient avidity to rescue them from a default apoptotic pathway termed "death by neglect." A small fraction of DP thymocytes express a TCR with sufficient avidity to respond to self. Thymocytes with TCRs having high avidity for self exceeding a certain threshold are eliminated by apoptosis (negative selection). Thymocytes bearing TCRs with intermediate avidity for self are rescued from death by neglect and differentiate into $CD4^+$ or $CD8^+$ single-positive (SP) cells and migrate to the periphery (secondary lymphoid organs and other tissues), a process known as positive selection (1, 2). The quantitative differences between avidity of the TCR for self translate into qualitative differences in the consequences of TCR signaling in DP thymocytes. For example, activated Erk associates with the plasma membrane during negative selection but remains in the cytoplasm during positive selection (3), and the adapter Themis appears to participate in positive but not negative selection (4–6). Thymocyte selection is responsible in large part for the development of a self-tolerant T cell repertoire capable of responding to foreign antigens.

Inherent in any model of thymocyte selection based on avidity for self is that environmental cues that affect TCR signaling have the potential to shift the avidity threshold that results in positive versus negative selection. One such possible cue is glucocorticoids, which are actually synthesized in the thymus (7). Although glucocorticoids are usually considered to be immunosuppressive, several lines of evidence have suggested that they may promote positive selection by antagonizing negative selection signals (7–9). To address the question of how endogenous glucocorticoids might influence the generation of an adaptive immune response, we created a conditional knockout mutant of the glucocorticoid receptor (GR; encoded by *Nr3c1*) and crossed it to *Lck-Cre* transgenic mice to delete the *GR* specifically in thymocytes ($GR^{lck\text{-}Cre}$ mice). We found that these mice are immunocompromised due to skewing of the T cell repertoire toward lower avidity for self because of negative selection of clones with TCRs in the range that normally leads to positive selection. Thus, endogenous glucocorticoids are required to ensure adaptive immune response fitness because they promote the selection of T cells that have sufficient affinity for self.

Results

Generation of conditional exon 3–targeted GR-knockout mice. To address the role of glucocorticoid signaling in T cell development and function, we generated mice in which exon 3 of *GR*, which encodes the DNA-binding domain, was flanked by *loxP* recombination sites, a strategy that has previously been used to target the GR (10). Early thymic deletion of *GR* was achieved by crossing floxed mice with mice expressing a *Cre* transgene driven by the *lck* proximal promoter, which is first expressed at the double negative 2 (DN2) stage of thymocyte development ($GR^{lck\text{-}Cre}$ mice) (11–13). Immunoblot analysis revealed that the GR was undetectablein purified $CD4^+$ thymocytes (DP and $CD4^+$ SP cells) from $GR^{lck\text{-}Cre}$ mice and was present at approximately one-third of WT levels in cells heterozygous for *GR* ($GR^{fl/+;lck\text{-}Cre}$) (Figure 1A). The finding that expression was less than 50% in hemizygous cells may be due to the fact that GR levels are upregulated in a feed-forward fashion by glucocorticoids (14). Characterization of thymocytes at different stages of development showed that GR protein is expressed at very low to undetectable levels in $GR^{lck\text{-}Cre}$ DP, $CD4^+$,

Conflict of interest: The authors have declared that no conflict of interest exists.
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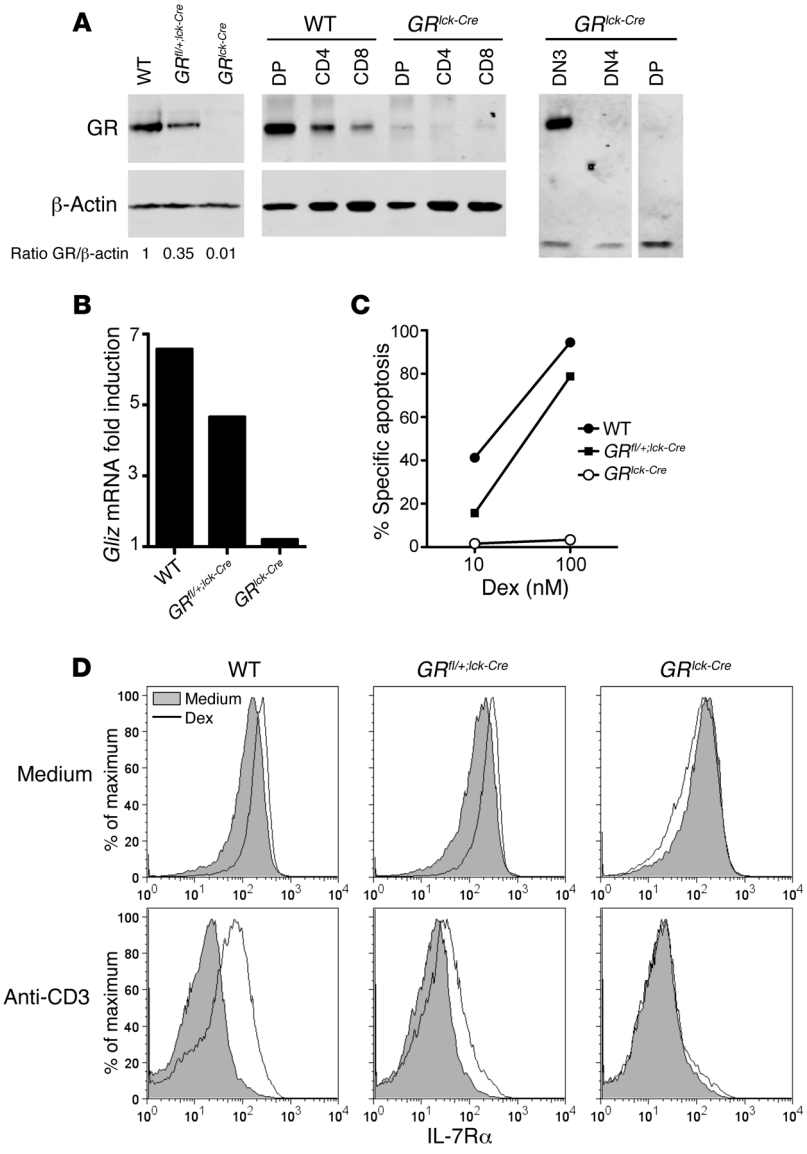

Figure 1
Physical and functional characterization of GR deletion. (**A**) Immunoblotting was performed to determine GR levels in (left panel) purified WT, heterozygous, and $GR^{lck\text{-}Cre}$ CD4$^+$ thymocytes; (middle panel) sorted WT and $GR^{lck\text{-}Cre}$ thymocytes; and (right panel) sorted DN3, DN4, and DP $GR^{lck\text{-}Cre}$ thymocytes (in the right panel, non-contiguous lanes from a single gel were rearranged as indicated by the white line). (**B**) $GR^{lck\text{-}Cre}$ T cells do not upregulate *Gilz* mRNA in response to Dex. Sorted T cells were treated or not with 100 nM Dex for 1.5 hours, and *Gilz* mRNA levels were quantitated by real-time PCR. (**C**) Glucocorticoids do not kill $GR^{lck\text{-}Cre}$ DP thymocytes. Thymocytes were incubated with the indicated concentrations of Dex overnight, then stained for CD4, CD8, and Annexin V as a marker of apoptosis, and the percent specific Annexin V positivity of CD4$^+$CD8$^+$ cells is shown. The data are representative of 3 independent experiments. (**D**) Glucocorticoids do not regulate IL-7Rα levels in $GR^{lck\text{-}Cre}$ T cells. Splenocytes were incubated overnight in the absence or presence of anti-CD3 in medium alone or with 100 nM Dex. Surface IL-7Rα expression on Thy-1$^+$ cells is shown. The data are representative of 3 independent experiments.

and CD8$^+$ SP cells, the loss occurring at the DN4 stage of development (Figure 1A). Antibodies against N- or C-terminal epitopes revealed no evidence of truncated GR products (data not shown). Functional deletion of the GR was assessed by three different means. Upon treatment with the synthetic glucocorticoid dexamethasone (Dex), mRNA encoding the glucocorticoid-induced leucine zipper protein (GILZ, encoded by *Tsc22d3*) — among the genes whose expression is increased most highly in glucocorticoid-treated cells (15) — was increased substantially in WT, less strongly in $GR^{fl/+;lck\text{-}Cre}$, and not at all in GR-deficient T cells (Figure 1B). In addition, whereas glucocorticoids induced WT DP thymocyte apoptosis in a dose-responsive manner, $GR^{lck\text{-}Cre}$ DP cells were completely resistant (Figure 1C). $GR^{fl/+;lck\text{-}Cre}$ thymocytes were sensitive to Dex, but modestly less responsive than WT cells. Finally, glucocorticoids upregulate expression of the IL-7Rα chain and antagonize the downregulation of that receptor caused by TCR-mediated activation (16). Both effects were abrogated in GR-deficient peripheral T cells, whereas $GR^{fl/+;lck\text{-}Cre}$ T cells displayed normal glucocorticoid-induced IL-7Rα upregulation but intermediate antagonism of activation-induced IL-7Rα downregulation (Figure 1D). Thus, GR protein expression and responsiveness to glucocorticoids was reduced in $GR^{lck\text{-}Cre}$ thymocytes and T cells in a gene dose–dependent fashion.

TCR proximal signaling induced by cross-linking is normal in $GR^{lck\text{-}Cre}$ T cells. The distribution of CD4$^+$ and CD8$^+$ T cells was unaffected by loss of GR expression, although T cell numbers were modestly lower (25%–35%) in the periphery (Figure 2A). The percentage of Tregs (CD4$^+$Foxp3$^+$) in thymus and spleen was similar in WT and GR-deficient mice (Supplemental Figure 2; supplemental material available online with this article; doi:10.1172/JCI63067DS1). There was little if any difference in IL-7Rα between WT, $GR^{fl/+;lck\text{-}Cre}$, and $GR^{lck\text{-}Cre}$ T cells, suggesting that in unperturbed mice circulating glucocorticoids do not have a substantial effect on this receptor (Figure 2B). There were no differences in the levels of TCR, CD4, or CD8 (P.R. Mittelstadt and J.D. Ashwell, unpublished observations) and no evidence of inappropriate T cell activation or perturbation of naive versus memory ratios as assessed by CD69, CD44, and CD62L staining.

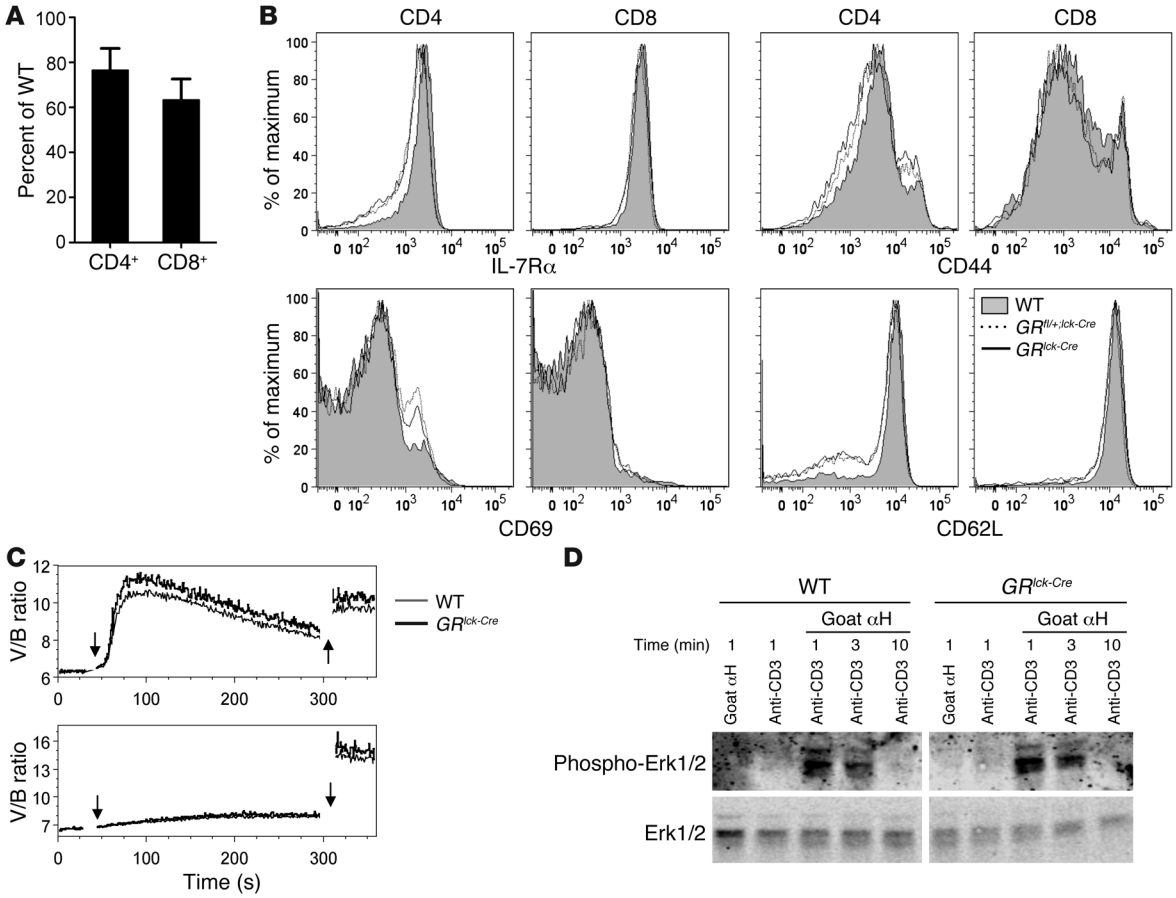

Figure 2

Normal proximal signaling in $GR^{lck\text{-}Cre}$ T cells. (**A**) Lymph node T cell counts of 8- to 12-week-old mice ($n = 7$), expressed as a percentage of WT. (**B**) Splenic T cell surface phenotype. (**C**) WT (CD45.1, light lines) and $GR^{lck\text{-}Cre}$ (CD45.2, heavy lines) splenocytes were labeled with Indo-1, coated (upper panel) or not (lower panel) with anti-CD3, washed, placed at 37°C, and collected with a flow cytometer. Goat anti-hamster (first arrow) and ionomycin (second arrow) were added at the indicated times. Intracellular Ca^{2+} was measured by plotting the violet/blue (V/B) ratio of Indo-1 fluorescence against time. (**D**) WT and $GR^{lck\text{-}Cre}$ T cells were coated with the indicated antibodies on ice, washed, and placed at 37°C in the presence or absence of cross-linking goat anti-hamster (αH). Lysates of cells harvested at the indicated times were immunoblotted for Erk1/2 and phospho-Erk1/2. Non-contiguous lanes from a single gel were rearranged. Data are from 1 of 3 experiments of the same design that gave similar results.

It has been reported that the GR associates with a TCR signaling complex that includes TCR, Lck, Fyn, and HSP90, and knockdown studies implicated the unliganded GR as a positive regulator of TCR-dependent Lck/Fyn activation (17, 18). It was proposed that upon binding glucocorticoids, the GR dissociates from the complex, resulting in impaired signaling, providing a non-genomic mechanism for GR inhibition of TCR-mediated activation. If so, one would expect to see impaired proximal signaling downstream of the TCR in $GR^{lck\text{-}Cre}$ T cells. We examined two critical early events that follow TCR cross-linking, Ca^{2+} flux and activation of the MAPK Erk. Anti-CD3–induced Ca^{2+} flux (Figure 2C) and Erk activation (Figure 2D) were unaffected by the absence of the GR. A late functional response, T cell proliferation, was also unaffected (see below). These results indicate that the unliganded GR does not play a major role in proximal TCR signaling.

Attenuated response of polyclonal $GR^{lck\text{-}Cre}$ T cells to antigen. The proliferative response of WT, $GR^{fl/+;lck\text{-}Cre}$, and $GR^{lck\text{-}Cre}$ T cells to a variety of stimuli was measured (Figure 3A). All three populations proliferated similarly when stimulated with PMA and ionomycin, which bypass the TCR, or with immobilized anti-CD3 in the presence of anti-CD28, a stimulus that is independent of TCR affinity for pMHC (Figure 3A). In contrast, the response of C57BL/6 $GR^{lck\text{-}Cre}$ T cells to allogeneic (H-2^a) antigen-presenting cells was markedly reduced (Figure 3A). Interestingly, the response of T cells heterozygous for the GR was blunted to the same extent as those lacking the GR.

Recognition of alloantigen by T cells is thought to be functionally similar to recognition of foreign antigen because CDR1 and CDR2 regions of the TCR interact with common determinants on polymorphic MHC molecules so that, as with foreign antigen, the principal interactions that promote cell activation are provided by foreign peptides (19, 20). We therefore asked whether conventional antigen-specific responses are affected by GR deficiency. C57BL/6 or B10.A mice were immunized with OVA or pigeon cytochrome *c* fragment 81–104 (PCC 81–104), respectively, and 8 days later the draining lymph node T cells were restimulated in vitro (Figure 3B). The recall response to both antigens was profoundly affected, there being an approximately 10-fold shift in the OVA dose-response

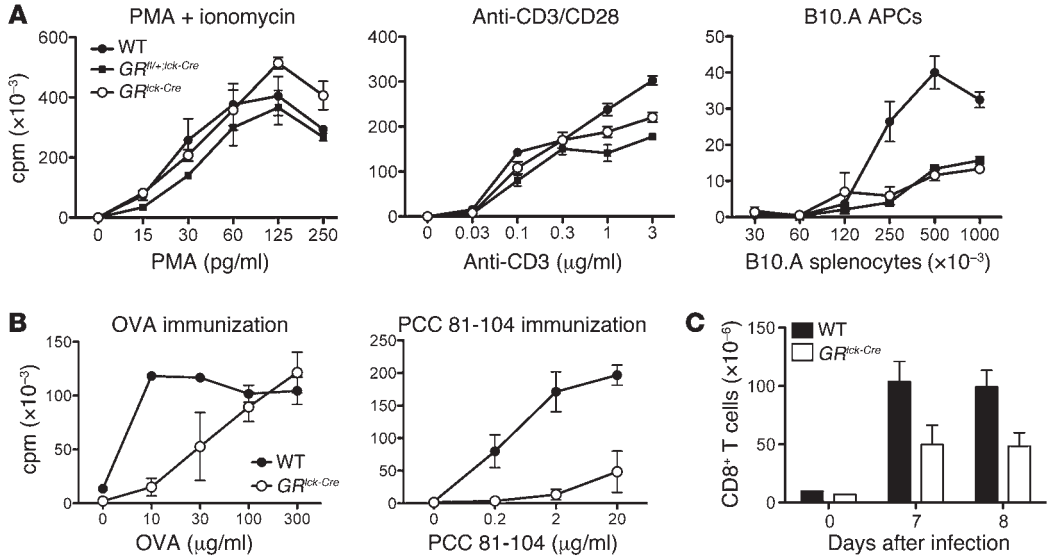

Figure 3

Reduced antigen-specific T cell frequency in $GR^{lck\text{-}Cre}$ T cells. (**A**) $GR^{lck\text{-}Cre}$ T cells proliferate normally to PMA plus ionomycin (P+I) and cross-linked CD3 (anti-CD3/CD28), but not to alloantigen. 5×10^4 (P+I or anti-CD3/CD28) or 1.5×10^5 (alloantigen) purified T cells of the indicated genotypes were cultured in triplicate with 1 μg/ml ionomycin and the indicated amounts of PMA, 1 μg/ml plate-bound anti-CD28 and the indicated amounts of plate-bound anti-CD3, or irradiated B10.A splenocytes in 96-well plates. After 48 (P+I or anti-CD3/CD28) or 72 (alloantigen) hours, wells were pulsed overnight with [^{3}H]thymidine and harvested. Data are shown as the mean cpm of triplicate cultures. Results are representative of 4 (P+I and anti-CD3/CD28) and 3 (alloantigen) independent experiments. (**B**) WT and $GR^{lck\text{-}Cre}$ C57BL/6 mice were immunized with 50 ng OVA in CFA (left panel) or WT and $GR^{lck\text{-}Cre}$ B10.A mice were immunized with 10 pmol PCC 81–104 in CFA (right panel). After 8–9 days, T cells from draining lymph nodes were incubated with 5×10^5 irradiated syngeneic splenocytes and the indicated concentrations of antigen for 4 days, pulsed overnight with [^{3}H]thymidine, and harvested. Each point is the average of 3 mice. (**C**) WT and $GR^{lck\text{-}Cre}$ B6 mice were infected with LCMV. At the indicated times after infection, splenic total CD8$^+$ T cells were analyzed. Data are shown as mean ± SEM (day 0, $n = 2$; day 7, $n = 3$; day 8, $n = 4$).

curve and a 100-fold shift in the PCC 81–104 response curve toward a requirement for more antigen for the same level of proliferation.

To evaluate an antigen-specific primary immune response, we infected mice with LCMV, which induces a massive CD8$^+$ T cell response that peaks on day 7–8 and declines thereafter (21). Whereas LCMV caused CD8$^+$ T cells in WT mice to expand to more than 10^8 in the spleen on day 7, the peak response in $GR^{lck\text{-}Cre}$ mice was reduced by half (Figure 3C). Thus, just as with the primary response to alloantigen, the primary antiviral response of T cells that developed in the absence of glucocorticoid signaling was attenuated.

The normal T cell response to TCR cross-linking but not antigen is consistent with an altered TCR repertoire. To test the possibility that other factors, such as the response to costimulation, might be responsible for the poor response to antigenic stimulation, we studied the responses of αβ TCR transgenic T cells, in which the affinity for antigen is fixed. Antigen dose-response analyses were performed with lymph node T cells from P14 (MHC I–restricted) and AND (MHC II–restricted) TCR transgenic mice stimulated in vitro with their corresponding peptide ligands, gp33–41 and PCC 81–104, respectively (Figure 4A). WT and $GR^{lck\text{-}Cre}$ TCR transgenic T cells responded identically, indicating that the lack of GR did not affect the ability of T cells to respond to physiologic pMHC via TCR occupancy. To more closely mimic the results obtained with immunization of mice with polyclonal T cells, equal numbers of congenically marked WT and $GR^{lck\text{-}Cre}$ P14 T cells were adoptively transferred into normal hosts, which were then immunized with gp33–41 in CFA. Eight to 11 days later, the draining lymph nodes were recovered and analyzed (Figure 4B). Although the numbers of transferred WT to $GR^{lck\text{-}Cre}$ P14 T cells were similar (input), 8–11 days after immunization, the ratio between these population was skewed toward $GR^{lck\text{-}Cre}$ P14 T cells (recovered), and on average approximately 3 times more $GR^{lck\text{-}Cre}$ P14 than WT T cells were recovered. The relative increase in the number of GR-deficient compared with GR-sufficient T cells probably reflects the former's insensitivity to the antiproliferative effects of circulating endogenous glucocorticoids. The recovered T cells were labeled with CFSE and stimulated in vitro with gp33–41 and analyzed 2 days later. The two populations proliferated equally at high antigen concentrations, and the $GR^{lck\text{-}Cre}$ P14 T cells actually proliferated better than WT cells at a low concentration, perhaps reflecting a greater degree of activation in vivo (Figure 4C). Similar results were obtained after 3 days of in vitro stimulation (data not shown). These results rule out the possibility that T cells lacking the GR die or become anergic after immunization. The greater expansion of $GR^{lck\text{-}Cre}$ T cells suggests that endogenous glucocorticoids limit the proliferation of normal T cells, making the poor response of polyclonal $GR^{lck\text{-}Cre}$ T cells to immunization even more striking.

Reduced tonic TCR signaling and competition for self pMHC by $GR^{lck\text{-}Cre}$ T cells. Peripheral naive T cell survival depends on tonic TCR signaling via recognition of self pMHC (22). These tonic signals correspond well with constitutive TCRζ tyrosine phosphorylation, a direct consequence of TCR occupancy (23). To assess functional avidity for self pMHC, we characterized the phosphorylation of TCRζ in freshly isolated T cells. TCRζ tyrosine phosphorylation was reduced 40%–50% in $GR^{lck\text{-}Cre}$ CD4$^+$ and CD8$^+$ compared with WT T cells (Figure 5A). Notably, there was no gene-dose effect,

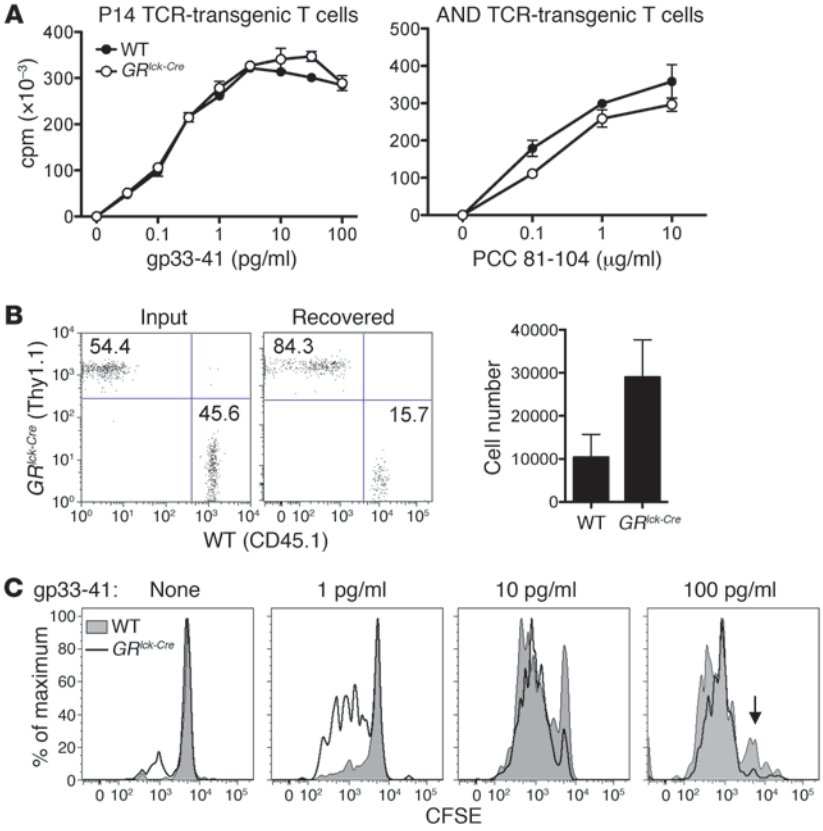

Figure 4
TCR-transgenic $GR^{lck\text{-}Cre}$ T cells proliferate normally when challenged with antigen in vitro and in vivo. (**A**) 5×10^4 B10.A WT or $GR^{lck\text{-}Cre}$ lymph node T cells from C57BL/6 P14 (left panel) or AND TCR mice (right panel) were incubated for 2 days with 5×10^5 irradiated WT C57BL/6 (P14) or B10.A (AND TCR) splenocytes in the presence of the indicated concentrations of antigen. (**B** and **C**) GR-deficient T cells have no intrinsic defect in ability to respond to antigen. (**B**) 5×10^5 WT (CD45.1$^+$) and $GR^{lck\text{-}Cre}$ (Thy1.1$^+$) P14 T cells were adoptively transferred into C57BL/6 mice (Input), which were immunized 2 days later with gp33–41 in CFA. After 8 days, draining lymph node cells were stained (Recovered) and counted, and recovery per draining (popliteal and inguinal) lymph node is shown. Numbers in the dot plots represent the percentages of CD8$^+$Vα2$^+$ cells in the corresponding quadrants. Data are shown as mean ± SEM (n = 3). (**C**) The recovered cells (shown in **B**) were labeled with CFSE and cultured with the indicated concentrations of gp33–41. CFSE dilution after 48 hours in culture is shown in the lower panels. The CFSE peak of undivided cells in the 100 pg/ml gp33–41 sample is indicated by an arrow.

the reduction in phospho-TCRζ in $GR^{fl/+;lck\text{-}Cre}$ T cells being nearly the same as in $GR^{lck\text{-}Cre}$ T cells. To determine whether this decrease was indeed the result of a decrease in tonic TCR signaling due to a repertoire with lower avidity for self, we evaluated the contribution of TCR diversity by examining constitutive TCRζ phosphorylation in AND αβ TCR T cells (Figure 5B). T cells from WT and $GR^{lck\text{-}Cre}$ AND mice had very similar phospho-TCRζ levels, indicating that when the receptor is fixed, tonic signaling is independent of GR expression. These results imply that the polyclonal TCRs expressed in non-transgenic mice are of lower avidity for self.

T cells compete with one another in vivo for access to self pMHC, and those with higher-affinity receptors have a survival advantage (24). Congenically marked WT and $GR^{lck\text{-}Cre}$ lymph node T cells were mixed together at an approximately 1:1 ratio and transferred into otherwise unmanipulated C57BL/6 mice. As shown in Figure 5C, after 4–7 weeks the number of WT T cells recovered was almost twice that of $GR^{lck\text{-}Cre}$ T cells. Thus, and consistent with the decrease in TCR-mediated tonic signaling, $GR^{lck\text{-}Cre}$ T cells competed less well for self pMHC than WT T cells in vivo.

Thymocyte development in $GR^{lck\text{-}Cre}$ mice. We addressed the effect of glucocorticoid unresponsiveness on thymocyte development by characterizing thymus size and composition. Total thymus cellularity was reduced by approximately 45% in young $GR^{lck\text{-}Cre}$ mice relative to WT controls. The difference was due to reductions in DP and mature SP cell numbers (Figure 6A). The number of DN and immature CD8$^+$ cells (ISP: CD8$^+$TCR^{lo} cells that precede the DP stage, ref. 25) was comparable (Figure 6A, inset). There were no differences in TCR levels between WT and $GR^{lck\text{-}Cre}$ thymocytes in the various subsets (P.R. Mittelstadt and J.D. Ashwell, unpublished observations). The distribution of DN1–DN4 cells, based on CD44 and CD25 expression, was similar in $GR^{lck\text{-}Cre}$ and WT thymocytes (P.R. Mittelstadt and J.D. Ashwell, unpublished observations). The decrease in thymocyte number first seen at the DP stage could have two non–mutually exclusive causes: a block in the ISP to DP transition and/or increased sensitivity to TCR-mediated deletion. To evaluate the contribution of TCR signaling, we analyzed thymocyte development in $GR^{lck\text{-}Cre}$ cells lacking TCRα. *Tcra*$^{-/-}$ thymocytes express the pre-TCR and can progress to the DP stage, but because they lack mature αβ TCRs cannot progress beyond this point (26). $GR^{lck\text{-}Cre}$ *Tcra*$^{-/-}$ DP thymocytes were reduced by approximately 20% (Figure 6B), compared with 45% in $GR^{lck\text{-}Cre}$ mice that expressed αβ TCRs (Figure 6A), whereas DN and ISP numbers were normal. This result indicates that approximately half of the thymocyte reduction in $GR^{lck\text{-}Cre}$ thymocytes is TCR dependent, and the remaining reduction is apparently due to TCR-independent events occurring at or shortly after the ISP to DP transition.

The loss of DP thymocytes in an αβ TCR–dependent fashion is consistent with the notion that glucocorticoids blunt the effectiveness of TCR signaling for selection and that in the absence of the GR, some thymocytes that would normally undergo positive selection are instead deleted by negative selection. To directly test this, we used a genetic model in which the avidity of the TCR for self can be varied in a predictable manner and assessed the effect of GR expression on thymocyte selection. $GR^{lck\text{-}Cre}$ mice were crossed with mice transgenic for the AND αβ TCR, a receptor that recognizes PCC 81–104 presented by MHC II I-E^k (encoded by *H2-Eb1*). Selection of the AND TCR is strongly dependent on the MHC: it is positively selected on the H-2^b haplotype (lacking I-E), positively and negatively selected on the H-2^a haplotype (expressing I-E^k), and not positively selected on the H-2^q haplotype (27, 28). To ensure that

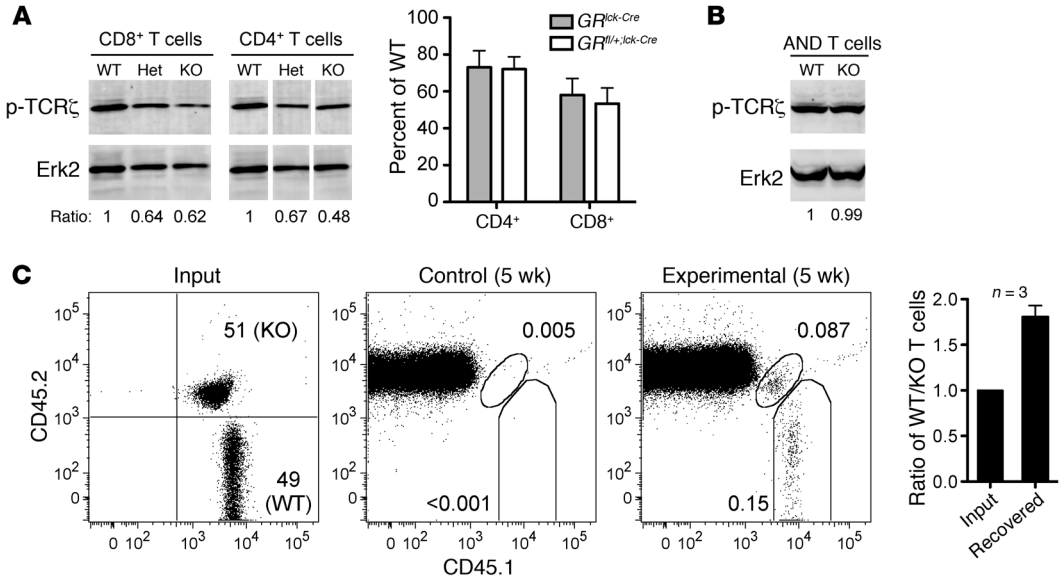

Figure 5

Reduced tonic TCR signaling in naive $GR^{lck\text{-}Cre}$ T cells. (**A**) Left panel: Steady-state phosphorylation of TCRζ in $GR^{lck\text{-}Cre}$ (KO) and $GR^{fl/+;lck\text{-}Cre}$ (Het) normal T cells is reduced. Immunoblot analysis of TCRζ phosphorylation and total Erk2 in purified peripheral CD4+ and CD8+ T cells. The level of TCRζ phosphorylation, normalized to Erk2, is shown below as a percentage of WT. For CD4+ T cells, noncontiguous lanes from a single gel were rearranged. Right panel: TCRζ phosphorylation, expressed as a percentage of WT, averaged from 2 ($GR^{fl/+;lck\text{-}Cre}$) and 3 ($GR^{lck\text{-}Cre}$) independent experiments. (**B**) Steady-state phosphorylation of TCRζ in $GR^{lck\text{-}Cre}$ and WT AND TCR CD4+ T cells. (**C**) Reduced long-term survival of adoptively transferred $GR^{lck\text{-}Cre}$ T cells. WT (CD45.1+) and $GR^{lck\text{-}Cre}$ (CD45.1+CD45.2+) lymph node cells were mixed together and transferred i.v. into C57BL/6 (CD45.2+) recipients. One representative experiment of 3 is shown, with CD45.1 versus CD45.2 staining of Thy1.2+ cells that were transferred (Input) and the cells recovered from the spleen 5 weeks later (center panels). Nonspecific staining in a CD45.2+ mouse (control) was used to calculate cell recovery. Numbers in the dot plots represent the percentages of Thy1.2+ cells. The average of 3 independent experiments in which cells were recovered and analyzed from 4–7 weeks after transfer is shown in the right panel.

all thymocytes expressed the AND αβ TCR, we also backcrossed the mice to animals deficient in recombination activating gene 2 (RAG-2). Examination of thymi from 4-week-old MHC-congenic animals revealed a direct correlation between strength of TCR signaling and the effect of GR expression on thymocyte development (Figure 6C and Supplemental Figure 1). In comparison to WT AND mice, $GR^{lck\text{-}Cre}$ DP cell numbers were decreased approximately 25% in non-selecting H-2^q AND mice, similar to those in mice lacking αβ TCR expression (Figure 6B). When the AND TCR was expressed in the positively selecting H-2^b haplotype, absence of the GR caused a 50% reduction of DP cells compared with MHC-matched GR-expressing WT cells, and introduction of one I-E^k (H-2^b/H-2^a) or two I-E^k alleles (H-2^a) markedly increased this difference, resulting in an 82% or 95% decrease in DP cells, respectively. Thus, increasing the strength of selection signals (H-2^q < H-2^b < H-$2^{b/a}$ < H-2^a) in the absence of GR resulted in progressively greater deletion of AND TCR DP thymocytes (Figure 6C).

Differential TCR Vβ CDR3 use in WT and $GR^{lck\text{-}Cre}$ T cells. To directly assess the effect of GR expression on thymocyte selection, we determined the amino acid sequences of the TCR CDR3 regions, which are largely responsible for contact with peptide in the MHC groove (29), from GR-deficient and GR-sufficient T cells. We chose to examine Vα11Vβ3-bearing cells, which dominate the MHC II–restricted response to PCC in B10.A mice (30, 31). Naive Vα11+Vβ3+ CD4+ T cells were sorted from 3 WT mice and 3 $GR^{lck\text{-}Cre}$ B10.A mice and their TCRβ regions subjected to high-throughput sequencing (32). There was no difference between the WT and $GR^{lck\text{-}Cre}$ samples in the average size or distribution of CDR3 lengths. The relative use of Jβ regions in Vβ3 CDR3s is shown in Figure 7A. Strikingly, 5 of the 10 Jβ regions were found to be used at statistically significantly different frequencies in the TCRβ chain of Vα11+Vβ3+ T cells from WT versus $GR^{lck\text{-}Cre}$ mice. We compared WT and $GR^{lck\text{-}Cre}$ Vβ3 CDR3 regions by examining amino acid sequences common to all three members of each group. There were 117 such CDR3 sequences from WT and 118 from $GR^{lck\text{-}Cre}$ mice, accounting for 2.8% and 2.7% of unique sequences, and 12.8% and 11.9% of the total number of reads, respectively. The degree to which the distributions of the differences of the mean frequencies of the WT and $GR^{lck\text{-}Cre}$ Vβ3 CDR3 sequences approximated a normal distribution was evaluated (Figure 7B). By this method, the groups were found to be significantly different from each other (WT, $P < 0.0003$; $GR^{lck\text{-}Cre}$, $P < 0.015$). Therefore, lack of GR expression during thymocyte selection results in alterations of the naive peripheral TCR repertoire.

Discussion

One of the most widely appreciated roles of glucocorticoids is their inhibitory effect on the transcription of genes involved in immune cell effector functions, for which they are widely used in the treatment autoimmune and inflammatory diseases. The physiological role of endogenous glucocorticoids in immunity, however, is not well understood. Based upon the observation that thymic epithelial cells produce glucocorticoids, we have previously explored their effect on T cell development using a number of indirect experimental models, including pharmacological inhibition

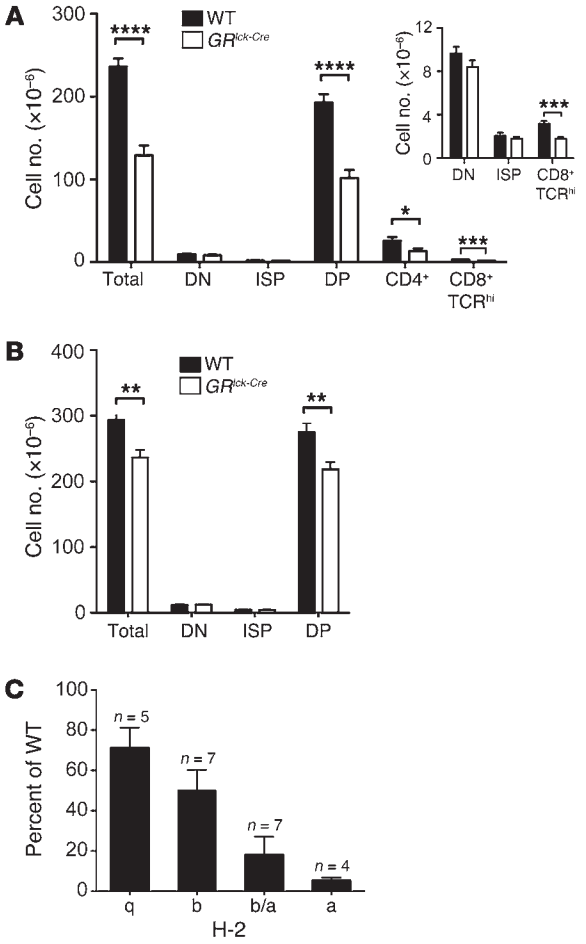

Figure 6
Thymus cellularity. (**A**) Cellularity in 4- to 6-week-old WT and $GR^{lck\text{-}Cre}$ thymi (n = 12). DN, mature $CD8^+$ ($CD8^+TCR^{hi}$), and ISP ($CD8^+TCR^{lo}$) cells are shown in the inset for clarity. (**B**) Cellularity in 4- to 6-week-old TCRα-deficient WT and $GR^{lck\text{-}Cre}$ thymi (n = 24). (**C**) Increased negative selection by GR deficiency. DP cell numbers from 4-week-old AND TCR $GR^{lck\text{-}Cre}$ thymi expressed as the percentage (mean ± SEM) of WT in $H\text{-}2^q$, $H\text{-}2^b$, $H\text{-}2^{b/a}$, and $H\text{-}2^{a/a}$ mice ± SEM. The number of pairs of WT and $GR^{lck\text{-}Cre}$ thymi examined for each genotype is indicated. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

of glucocorticoid production in fetal thymic organ culture and use transgenic mice expressing antisense GR targeted to thymocytes and T cells (7–9, 33, 34). The data obtained in these studies indicated that endogenous glucocorticoids shift the threshold between positive and negative selection to permit survival and maturation of thymocytes, with TCRs having higher avidity for self pMHC. In the GR-knockdown mice, in particular, the thymus was decreased in size for two reasons: a partial block in DN to DP development and increased antigen-specific negative selection of DP thymocytes. In an alternative gain-of-function approach, a rat transgenic for a hyperactive mutant GR displayed evidence of a changed repertoire, with altered selection of certain TCR Vβ genes and an altered TCRβ CDR3 spectratype profile (35). However, a study using mice in which the *GR* exon 2 was targeted, resulting in apparent *GR* deletion, found no obvious difference in thymocyte numbers, which was interpreted as a formal demonstration that glucocorticoids had little if any influence on thymocyte development (36). Subsequent studies revealed that deletion of exon 2 resulted in the use of a start site in exon 3 and the production of a truncated GR that contained the dimerization, DNA-binding, and ligand-binding domains, and in fact thymocytes from such animals responded to glucocorticoids by up- or downregulating many of the same genes that are regulated by the full-length receptor (37, 38). Another mouse in which *GR* exon 2 was conditionally targeted had a slight reduction in thymus size with no obvious abnormalities in thymocyte ratios, but selection was not directly addressed (39). True deletion of *GR* was achieved by conditionally targeting exon 3, which encodes the DNA-binding domain (10), which when deleted with Cre under the control of the Lck proximal promoter resulted in reportedly normal thymocyte development and T cell proliferation (the stimulus was not reported) (40). T cell selection has not been analyzed in mice in which *GR* exon 2 (39) or exon 3 (10) was targeted. Because of the apparent disagreement between experimental models, the current view is that the GR is dispensable for physiologic thymocyte development (41). However, the importance, if any, of endogenous glucocorticoids in thymocyte selection and the establishment of an effective immune system has not been tested in a rigorous genetic model in which thymocytes cannot respond to glucocorticoids.

We generated such a genetic model by targeting *GR* exon 3. Glucocorticoids act by binding the GR or its alternatively spliced isoforms, which all contain the DNA-binding domain encoded in part by exon 3 (42). By confining GR loss to cells of the T lineage, it was possible to address the role of glucocorticoids in thymocyte development and peripheral T cell responses in detail. The most striking finding was that despite the fact that GR-deficient T cells are resistant to the immunosuppressive effects of glucocorticoids, polyclonal T cells from $GR^{lck\text{-}Cre}$ mice responded poorly to antigenic stimulation, in vitro and in vivo. This was not due to an intrinsic defect in responsiveness, but rather to an altered T cell repertoire due to the TCR-dependent negative selection of cells that would ordinarily undergo positive selection. An interesting issue addressed in this report is the question of how recognition of self pMHC relates to recognition of foreign antigen. It was originally thought that randomly generated TCRs selected on self pMHC have an unbiased chance of recognizing a given foreign peptide antigen presented by self MHC. However, direct experimental examination of this question revealed that the strength of self-reactivity is directly related to TCR affinity for foreign antigen/MHC. Manipulations that decreased tonic TCR signaling, as evidenced by reduction of constitutive TCRζ phosphorylation, or the study of cells with naturally occurring variations in the level of TCRζ phosphorylation, revealed that lower TCRζ phosphorylation correlated with diminished responses to foreign antigens (23, 43). Similarly, $CD4^+$ memory T cells deprived of contact with MHC II had reduced responsiveness when reexposed to antigen (44). Although the mechanism is not completely understood, it has been suggested that tonic subthreshold stimulation leads to the concentration of signaling components and promotes TCR clustering, which would facilitate responses to stronger ligands (23). Our finding that the decrease in TCR avidity for self pMHC, resulting from the negative selection of cells bearing TCRs with relatively higher avidity for self pMHC, resulted in hyporesponsiveness to foreign antigens and alloantigen is consistent with these observations.

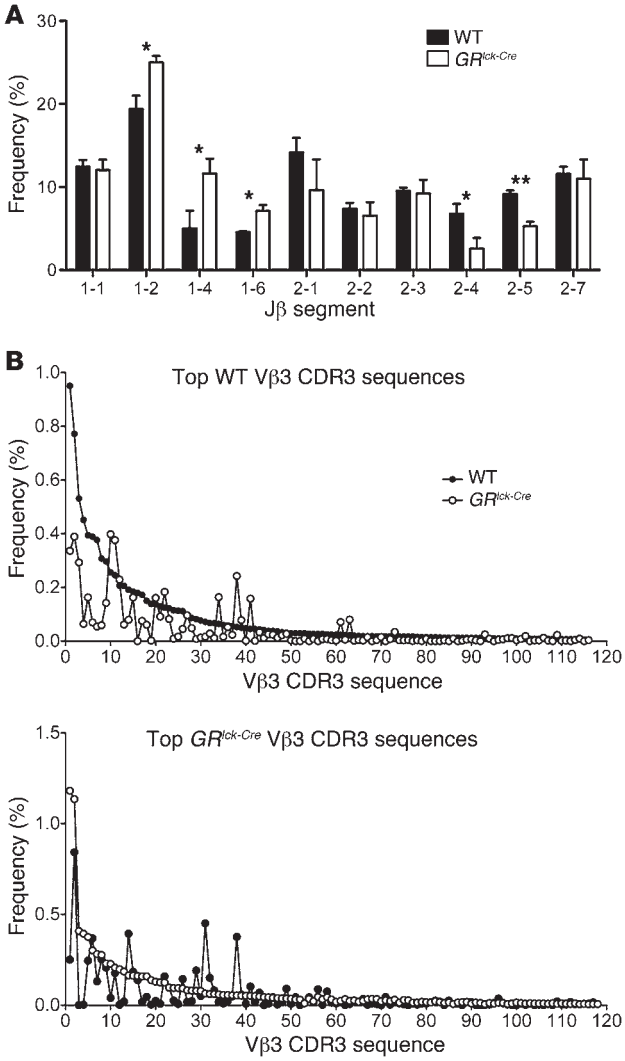

Figure 7
Sequence analysis of the CDR3 repertoires of naive Vα11$^+$Vβ3$^+$CD4$^+$ T cells from B10.A WT and *GR*$^{lck\text{-}Cre}$ mice. (**A**) Differential Jβ segment use by naive T cells. Averaged percentage of Jβ segment use in unique Vβ3 amino acid sequences in each group (n = 3). The data are shown as mean ± SEM. (**B**) CDR3 amino acid sequences common to all 3 WT or *GR*$^{lck\text{-}Cre}$ mice do not overlap. Vβ3 CDR3 sequences of naive Vα11$^+$Vβ3$^+$CD4$^+$ T cells common to each of 3 WT (upper panel) or *GR*$^{lck\text{-}Cre}$ (lower panel) mice were plotted in order of frequency. One sequence common to all 6 mice (CASSPGTANS), accounting for approximately 1% of the total, was omitted for clarity but included in statistical analyses. *P < 0.05, **P < 0.005.

Naturally occurring Tregs are thought to be derived from thymocytes that have relatively high affinity for self pMHC (45). That the frequencies of CD4$^+$Foxp3$^+$ cells were unchanged in the absence of the GR (Supplemental Figure 2) may reflect a balance between the loss of the highest-affinity Tregs and the recruitment of cells that would otherwise have become conventional CD4$^+$ T cells. The source of the glucocorticoids that regulate thymocyte selection is an open question. Whether they are produced locally and act in a paracrine fashion (46), or systemically and act in an endocrine manner, cannot be determined from the present study. Genetic models to specifically address these questions are being generated.

How do glucocorticoids regulate the avidity threshold between positive and negative selection? One potential mechanism would be by directly dampening proximal TCR signaling. Indeed, there are data suggesting that the unliganded GR is a component of the TCR signaling apparatus that is displaced when bound by glucocorticoids (17, 18). This is unlikely to explain our results, however, because TCR proximal signaling was found to be normal in GR-deficient T cells. Another possibility is that glucocorticoids may regulate the expression of genes specifically involved in positive selection. Recently, the zinc finger–containing protein schnurri-2 (Scn2; encoded by *Hivep2*) was shown to be required for optimal positive selection of thymocytes through its suppression of the mitochondrial death pathway (47), presumably by interacting with Bax, a target of the proapoptotic molecule Bim involved in thymocyte negative selection (48). Like *Bim* (encoded by *Bcl2l11*) (49), *Scn2* mRNA is upregulated in thymocytes by TCR signals (47). Whereas we also found that *Scn2* mRNA was upregulated by stimulation with PMA and ionomycin, glucocorticoids had no effect on *Scn2* mRNA levels, suggesting that glucocorticoid signaling does not act through modulation of *Scn2* (P.R. Mittelstadt and J.D. Ashwell, unpublished observation). Expression of *Gli1* mRNA, a target of hedgehog signaling in thymocytes and implicated in thymic selection (50), was also not affected in thymocytes treated with glucocorticoids (P.R. Mittelstadt and J.D. Ashwell, unpublished observations). A variant of this second model is that the effects of glucocorticoids on selection thresholds are simply due to their well-recognized ability to up- or downregulate many genes involved in T cell activation, which in the periphery is manifested as immunosuppression. We favor the notion that glucocorticoids, primarily by transcriptional interference with transcription factors such as AP-1, NF-κB, Stat5, and others (51–53) inhibit key events required for some aspects of thymocyte activation at the nuclear level. Thus, loss of glucocorticoid responsiveness would shift the positive/negative threshold to the left and enhance negative selection. It is an interesting question whether glucocorticoids regulate the window between death by neglect and positive selection. If they do, one might expect to see TCRs with normally subthreshold avidity for self being positively selected and contributing to the peripheral repertoire. We saw no functional evidence of new specificities, however, because the response of *GR*$^{lck\text{-}Cre}$ T cells to complex antigens such as OVA, LCMV, and alloantigen was always blunted. This can be understood if one considers that there is a minimum TCR avidity required to activate thymocytes. It has been proposed that avidities must be high enough for a sufficient "dwell time" of pMHC with the TCR to allow assembly of a complete TCR/coreceptor signaling complex, and avidities below this threshold do not induce sufficient proximal signaling to activate the cell (3, 54, 55). In such a case, glucocorticoids would have no effect, because they act distally to proximal TCR signaling. Studies to directly evaluate this possibility are being pursued.

An effector protein implicated in negative selection that is subject to repression by glucocorticoids is the orphan nuclear receptor Nur77 (56, 57), the expression of which correlates with thymic TCR signaling intensity (58). Although thought to mediate negative selection through its transcriptional regulatory properties (59), Nur77 can also translocate to mitochondria, where it blocks the antiapoptotic property of Bcl-2, a target of Bim, by inducing a conformational change (60). Preliminary experiments have sug-

gested that Nur77 (encoded by *Nr4a1*) and *Bim* mRNA levels are increased in $GR^{lck\text{-}Cre}$ thymocytes (P.R. Mittelstadt and J.D. Ashwell, unpublished observations). Another gene that is markedly upregulated by glucocorticoids in thymocytes and T cells is *Gilz*, which encodes a small leucine zipper–containing protein with transcriptional repressive properties and may amplify the suppressive effects of glucocorticoid signaling (61). Transgenic overexpression of GILZ reduced the number of DP thymocytes and decreased apoptosis induced by TCR cross-linking. Antigen-specific selection was not examined in these reports (62, 63).

The ability of glucocorticoids to induce thymocyte apoptosis, upregulate *Gilz* mRNA, and regulate IL-7Rα expression on T cells with only one *GR* allele was intermediate between WT and GR-deficient cells, typical of a gene-dose effect. In contrast, GR-hemizygous T cells had reduced tonic levels of phospho-TCRζ and responsiveness to stimulation by alloantigen that were comparable to those of cells lacking the GR. These observations suggest that there is a threshold for the effects of glucocorticoid on antigen-specific thymocyte selection. Such thresholds have been reported for Dex-induced apoptosis of human T and pre-B cell lines (14) and in the EAE model, in which T cell GR haploinsufficiency rendered mice completely refractory to glucocorticoid therapy (64). In the former case, threshold effects were attributed to GR auto-upregulation. Consistent with this, we found GR levels to be reduced by more than half in GR-heterozygous thymocytes. Threshold effects can also occur if there is no signal amplification downstream of the liganded GR, meaning that it directly mediates its functions by binding DNA or other transcriptional regulators, and thus a minimum number of receptors may need to be available to effect particular biologic activities. Interestingly, the GR antisense transgenic mice previously reported to affect thymocyte selection expressed approximately half of the normal level of GR (8).

Many of the models that have been proposed to account for ligand-specific discrimination between positive and negative selection invoke differences in the intensity or quality of TCR-proximal signaling due to the affinity and/or off-rate of the selecting peptide or differences in the kinetics of signaling (65, 66). These models imply that the intensity or quality of proximal signals downstream of TCR occupancy determine the physiologic outcome: survival and maturation or death. That glucocorticoid signaling can convert one outcome to another (negative to positive selection) provides a means of regulating antigen-specific selection independently of proximal TCR signaling.

Methods

Mice. C57BL/6 and congenic strains, $Rag2^{-/-}$, $Tcra^{-/-}$, AND TCR-transgenic (67), and *Lck-Cre*–transgenic mice (68) were obtained from The Jackson Laboratory, and P14 TCR-transgenic mice (69) were from Taconic. *Nr3c1* exon 3–targeted mice were generated by a strategy similar to that used by Tronche et al. (10). The 5′ *loxP* site was inserted into the EcoR1 site 0.1 kb upstream of exon 3, and a second *loxP* site was inserted into a BamHI site 1.8 kb downstream using an *Frt*-flanked *Neo* cassette. The Neo cassette was excised by crossing mice bearing the targeted allele with mice transgenic for the Flp recombinase driven by the β-actin promoter. Genotyping of recombinant animals was verified by Southern blotting of the first generation and subsequently by PCR of tail DNA. All mice used in this study were backcrossed for at least 6 generations onto the C57BL/6 background.

Flow cytometry. Lymphoid cells were collected and washed in PBS/2% FCS, FcγRII/III receptors were blocked with 2.4G2 (BD Biosciences), and the appropriate antibodies were used for surface staining. Cells were acquired on an LSRII or a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star). Surface IL-7Rα was detected using biotin-conjugated clone A7R34 (eBioscience). Intracellular Foxp3 was detected using the Mouse Regulatory T Cell Staining Kit from eBioscience. PE-conjugated Annexin V was obtained from BD Biosciences and CFSE from Invitrogen. Percent specific Annexin V^+ was calculated as ([experimental Annexin V^+ – spontaneous Annexin V^+]/[100 – spontaneous Annexin V^+]) × 100.

Ca^{2+} flux. WT ($CD45.1^+$) and $GR^{lck\text{-}Cre}$ ($CD45.2^+$) splenocytes were labeled together with 50 μM Indo-1 (Invitrogen) for 15 minutes at 37°C, washed in complete medium, and then incubated on ice with fluorescent antibodies with or without 10 ng/ml unlabeled anti-CD3 (clone 2C11; BD Biosciences). After warming to 37°C, cells were analyzed by flow cytometry for 30–40 seconds, at which time 3 μM goat anti-hamster (Jackson ImmunoResearch Laboratories Inc.) was added as a cross-linker, followed 3–4 minutes later by 2 μM ionomycin to verify Indo-1 loading. Relative Ca^{2+} concentration in $Thy1.2^+$ cells was determined by plotting the ratio of Ca^{2+}-bound to free dye. Data were analyzed with FlowJo software.

Real-time PCR. Total RNA was isolated with an RNeasy Mini kit (QIAGEN), and cDNA was generated with Superscript RT (Invitrogen). Real-time PCR was performed with SYBR Green PCR mix (Applied Biosystems) using previously described primers (38) and a 7500 Real-Time PCR system (Applied Biosystems). The results are relative to *Hprt* expression.

Flow sorting and T cell purification. Thymocytes were stained with anti-CD8 (clone 53-6.7) and anti-CD4 (RMA4-5). For purification of DN subpopulations, thymocytes were also stained with anti-TCRβ (H57), anti-CD25 (PC61.5), and anti-CD44 (IM7), and cells to be excluded (the dump channel) were stained with FITC-labeled antibodies to CD11b (M1/70), CD11c (HL3), CD3ε, NK1.1 (PK136), and TCRγδ (GL3). After excluding the cells in the dump channel, $CD4^-CD8^-TCR\beta^-$ thymocytes were identified as $CD25^+CD44^-$ (DN3) and $CD25^-CD44^-$ (DN4). For repertoire analysis, CD62L (MEL-14)$^+CD44^{lo}V\alpha11^+V\beta3^+CD4^+$ cells were sorted from spleen and lymph nodes. All sorts were performed with a FACSAria (BD Bioscience). For analysis of *Gilz* mRNA levels, $Thy1.2^+TCR\beta^+$ cells were sorted from lymph nodes.

Immunoblotting. To evaluate GR levels, we lysed cells in sample buffer and immunoblotted with antibodies recognizing β-actin (Sigma-Aldrich) and either the N terminus (M-20; Santa Cruz Biotechnology Inc.) or C terminus (PA1-815; Affinity Bioreagents) of the GR. To detect phosphorylated Erk1/2, T cells were lysed in 1% Triton X-100 with protease and phosphatase inhibitors and immunoblotted with anti–phospho-Erk1/2 (Thr202/Tyr204) and total Erk1/2 (L34F12; both from Cell Signaling Technology). To quantify partial tyrosine phosphorylation of the TCR ζ chain, $CD4^+$ and $CD8^+$ T cells obtained by negative selection (MACS; Miltenyi), were lysed in 1% NP-40 and immunoblotted for TCRζ phospho-Tyr142 (K25-408.69; BD Biosciences) and Erk2 (C14; Santa Cruz Biotechnology Inc.) under non-reducing conditions as described previously (70). Quantitation was done with an Odyssey Scanner (LI-COR).

Immunization and cell culture. Mice were immunized in the footpad with 8 pmol PCC 81–104, 5 pmol gp33–41 (both from New England Peptide), or 25 μg chicken egg OVA (Sigma-Aldrich) emulsified in CFA (Difco). For in vitro recall assays, 10^5 T cells from draining lymph nodes enriched by removing B cells with anti-mouse IgG-couple magnetic beads (PerSeptive Systems) were incubated with 5×10^5 irradiated (25 Gy) spleen cells and the indicated concentrations of antigen or numbers of irradiated allogeneic spleen cells in a total volume of 200 ml RPMI 1640 (Biofluids) supplemented with 10% heat-inactivated charcoal/dextran-treated fetal calf serum (HyClone), 100 mg/ml gentamicin, 4 mM glutamine, and 50 μM 2-mercaptoethanol (complete medium). After 48 hours (PMA and ionomycin or anti-CD3/anti-CD28) or 96 hours (antigen, alloantigen), the wells were pulsed with 1 μCi [^{3}H]thymidine and 18 hours later harvested; the incor-

poration of radioactivity was determined by liquid scintillation counting.

Adoptive transfer, immunization, and proliferation. Thy1.1$^+$ ($GR^{lck\text{-}Cre}$) and CD45.1$^+$ (WT) P14 TCR transgenic T cells (5×10^5) were purified by negative selection (STEMCELL Technologies), mixed at a 1:1 ratio, and injected i.v. into 2- to 3-month-old C57BL/6 recipients. Two days later, recipients were immunized with gp33–41 in CFA. After 8 or 11 days, draining lymph nodes were harvested, labeled with CFSE, and cultured in complete medium at a density 2.5×10^6 cells/ml and stimulated with gp33–41. CFSE dilution in CD45.1$^+$ or Thy1.1$^+$ TCRβ$^+$CD8$^+$Vα2$^+$ cells was analyzed by flow cytometry.

Adoptive transfer for analysis of survival. Three million WT (CD45.1) or $GR^{lck\text{-}Cre}$ (CD45.1/CD45.2) lymph node cells from littermates that were backcrossed for 9 generations to C57BL/6 mice were mixed and injected i.v. into 2- to 3-month-old male C57BL/6 recipients. Five (transfer 1), 4 (transfer 2), and 7 (transfer 2) weeks after transfer, splenocytes stained with antibodies to CD45.1, CD45.2, and Thy1.2. Relative recovery was calculated by subtracting background events appearing in the CD45.1$^+$CD45.2$^+$ and CD45.1$^+$CD45.2$^-$ gates of Thy1.2-gated T cells from unmanipulated C57BL/6 splenocytes, and the results were then normalizing to the ratios of input T cells (the $GR^{lck\text{-}Cre}$/WT ratio was 1.04 for experiment 1 and 1.1 for experiment 2).

Viral infection. The Armstrong strain of LCMV was a gift from Rafi Ahmed (Emory University School of Medicine, Atlanta, Georgia, USA). Mice were inoculated intraperitoneally with 2×10^5 PFU in 500 μl PBS.

Sequencing TCRβ CDR3 regions and statistical analysis. Genomic DNA from 11×10^4 to 62×10^4 sorted CD44^{lo}CD62L^{hi}Vα11$^+$Vβ3$^+$CD4$^+$ T cells from 3 WT and 3 $GR^{lck\text{-}Cre}$ B10.A mice was extracted, amplified, and sequenced individually by Adaptive TCR Corp. using the ImmunoSEQ procedure, in which all possible rearranged genomic TCRβ CDR3 sequences are amplified in a multiplex PCR reaction (32). CDR3 sequences were identified as described previously (32). For the analysis of the commonly used CDR3 sequences, unique TCR Vβ CDR3 amino acid sequences common to all 3 WT or $GR^{lck\text{-}Cre}$ mice and present above a threshold of 0.005%, which was selected to reduce spurious sequences, were selected. To assess the difference between the groups, the distribution of the differences between the means for each sequence for each group was analyzed by a 1-tailed, 1-sample *t* test.

Statistics. 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.

Study approval. Animal housing, care, and research were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23. Revised 1985), and all procedures on animals performed in this study were approved by the National Cancer Institute Animal Care and Use Committee.

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