

Glucocorticoids Oppose Thymocyte Negative Selection by Inhibiting Helios and Nur77

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Glucocorticoid (GC) signaling in thymocytes shapes the TCR repertoire by antagonizing thymocyte negative selection. The transcription factors Nur77 and Helios, which are upregulated in TCR-signaled thymocytes, have been implicated in negative selection. In this study, we found that GCs inhibited Helios and, to a lesser extent, Nur77 upregulation in TCR-stimulated mouse thymocytes. Inhibition was increased by GC preincubation, and reductions in mRNA were prevented by a protein synthesis inhibitor, suggesting that GCs suppress indirectly via an intermediary factor. Upregulation of Helios in TCR-stimulated thymocytes was unaffected by deletion of Nur77, indicating Nur77 and Helios are regulated independently. Whereas CD4⁺ thymocytes are positively selected in wild-type AND TCR-transgenic B6 mice, loss of GC receptor expression resulted in increased negative selection. Correspondingly, Helios and Nur77 levels were elevated in TCR^{hi}CD4⁺CD8⁺ (TCR-signaled) thymocytes. Notably, deletion of Helios fully reversed this negative selection, whereas deletion of Nur77 had no effect on CD4⁺CD8⁺ cell numbers but reversed the loss of mature CD4⁺ thymocytes. Thus, Nur77 and Helios are GC targets that play nonredundant roles in setting the signaling threshold for thymocyte negative selection. *The Journal of Immunology*, 2019, 203: 2163–2170.

Selection of self-tolerant, yet Ag-responsive, T cells takes place in the thymus, where random rearrangements of genes encoding the Ag TCR- α and - β chains produce a unique receptor for each thymocyte (1). Thymocytes that express the coreceptors CD4 and CD8 (double-positive [DP] thymocytes) survey peptides derived from ubiquitously expressed self-antigens presented by major histocompatibility molecules (self-peptide/self-MHC [self-pMHC]) on cortical thymic epithelial cells (TECs). Those with TCRs that fail to recognize self-pMHC with sufficient affinity undergo “death by neglect.” Strong recognition of self-pMHC induces apoptotic death (negative selection), and the remaining thymocytes with intermediate affinities for self-pMHC survive (positive selection). Survivors of these screening steps in the cortex downregulate either CD4 or CD8 to become single-positive (SP) cells and express the homing receptor CCR7, which drives them into the medulla for a second round of screening against tissue-restricted self-antigens whose expression is driven by the autoimmune regulators Aire and Fezf2 (2). Tissue-restricted Ags are presented by medullary TECs and transferred to and cross-presented by dendritic cells. Tissue-restricted Ags can negatively select thymocytes or direct them into alternative agonist-selected

lineages such as regulatory T cells (Tregs), whose repertoires have relatively high affinity for self-pMHC (3). Thymocytes that survive this second selection step exit the thymus as naive effector or alternate-lineage T cells.

Glucocorticoids (GCs) are steroid hormones that cross the plasma membrane and bind to an intracellular receptor (GC receptor [GR]). The liganded GR alters gene transcription directly by binding to responsive elements in the DNA and indirectly by binding to and interfering with other transcription factors (4). Whereas GCs typically upregulate metabolic processes, they are well known to suppress production of inflammatory cytokines such as IL-1, IL-2, IL-4, IL-6, GM-CSF, TNF- α , and IFN- γ (5). We have shown that GCs are produced by TECs, and that the T cell repertoire, which refers to the diversity of the clonally expressed TCRs in an individual, is shaped by GR signaling in developing thymocytes (6–10). Although it has no effect on TCR signaling per se (9), GR signaling antagonizes events downstream of the TCR in the thymus, mitigating negative selection signals and allowing positive selection of cells bearing TCRs with relatively high affinity for self-pMHC. Targeted disruption of the GR in thymocytes results in increased negative selection and a “weakened” T cell repertoire as evidenced by diminished responses to foreign Ag, lymphocytic choriomeningitis virus infection, and allogeneic APC (9). In contrast, GR-deficient T cells responded normally when the repertoire was fixed by introducing Ag-specific $\alpha\beta$ TCR transgenes. Targeted disruption of *Cyp11b1*, the last enzyme in the series leading to corticosterone production, in TECs resulted in diminution of GC-driven gene expression in thymocytes and increased apoptosis of TCR-signaled DP cells (10, 11). Thus, GCs oppose TCR signals and rescue T cells with TCRs having relatively high avidity for self that would otherwise be negatively selected (5).

Although the molecular mechanism by which GCs affect Ag-specific thymocyte selection is unknown, given the GR’s well-established functions, it is likely to be through changes in transcription. Two potential targets are Nur77 (Nr4a1) and Helios (Ikzf2), transcription factors associated with thymocyte negative selection (12, 13). Nur77 transcriptional activity has been shown to be suppressible by GCs (14, 15). Nur77, whose upregulation in thymocytes correlates with strength of TCR signals (16, 17),

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Abbreviations used in this article: CHX, cycloheximide; DP, double-positive; EGFP, enhanced GFP; GC, glucocorticoid; GR, GC receptor; self-pMHC, self-peptide/self-MHC; SP, single-positive; TEC, thymic epithelial cell; Treg, regulatory T cell; WT, wild-type.

promotes Ag-induced apoptosis (12, 18–20). Whereas Nur77 has been proposed to act by converting mitochondrial Bcl-2 from an anti- to proapoptotic form (21), its ability to induce apoptosis was shown to depend on its transcriptional activity (22). Nur77 deficiency inhibited thymocyte negative selection in MHC class II– (23) and MHC class I–specific (24) TCR-transgenic models. Helios is upregulated in TCR-signaled CD4^{low}CD8^{low} DP thymocytes that recognize self-peptide/MHC ligands with high affinity and are destined to undergo negative selection (13, 25). Because Helios was not upregulated in thymocytes receiving intermediate strength TCR signals that induced positive selection, it was proposed to be specifically triggering during negative selection (13). The association of these transcription factors with negative selection led us to investigate the possibility that GCs might alter the TCR repertoire by regulating their activity.

Materials and Methods

Mice

C57BL/6 (B6), *Rag2*^{−/−}, and TCR-transgenic mice (26) were obtained from The Jackson Laboratory. AND mice were crossed with *Rag2*^{−/−} mice to prevent rearrangement of the endogenous *TCRα* locus. *Lck-Cre*–transgenic mice were obtained from Taconic Biosciences. *Nur77*^{−/−} mice (27) were obtained from C. Benoist. *Nr3c1* (GR) exon 3 conditionally targeted mice were described (9). *Helios* conditionally targeted (28) and enhanced GFP (EGFP) reporter (29) mice were kindly provided by A. Thornton. All mice used in this study were backcrossed for at least seven generations onto B6. Primer sequences used for genotyping are provided in Supplemental Table I.

Abs

Anti-CD3 (145-2C11), anti-CD28 (37.51), and anti-mouse CD16/CD32 (2.4G2) were purchased from Bio X Cell. Flow cytometry Abs against CD4 (RM4-5), CD69 (clone H1.2F3), Nur77 (12.14), CD25 (PC61.5), and PD-1 (J43) were from eBioscience; against CD8α (53-6.7) and TCRβ (H57) from BD Pharmingen; and against Helios (22F6) from BioLegend. LIVE/DEAD Fixable Blue Dead Cell Stain was from Invitrogen. Immunoblotting, Abs against Nur77 (12.14), Helios (D8W4X), and β-Actin (AC-15) were from eBioscience, Cell Signaling, and Sigma-Aldrich, respectively. PE-conjugated α-galactosylceramide–loaded mouse CD1d tetramer was obtained from the National Institutes of Health tetramer core facility.

Flow cytometry

Cells were incubated with primary Abs against CD16 and CD32 and LIVE/DEAD for 20 min at 4°C, washed in FACS buffer, then fixed with Foxp3 Transcription Factor Fixation Buffer (eBioscience). After washing with permeabilization buffer (eBioscience), fixed cells were incubated in 0.2 ml permeabilization buffer for 1 h at 4°C. After spinning cells out of permeabilization buffer, cells were incubated overnight at 4°C with Abs in permeabilization buffer. Data were obtained using BD LSRFortessa flow cytometers with FACSDiva software (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Reagents

Corticosterone and cycloheximide (CHX) were obtained from Sigma-Aldrich and Z-VAD-FMK from R&D Systems.

Cell culture

T cells were cultured in RPMI 1640 (Biofluids) supplemented with 10% heat-inactivated, charcoal-stripped FCS (Corning), 100 μg/ml gentamicin, 2 mM glutamine, and 50 μM 2-ME. Thymocytes were activated by incubation in 24-well or 12-well plates that were coated with 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28.

Immunoblotting

Cell samples were boiled in SDS sample buffer for 10 min, proteins separated by SDS-PAGE, transferred to nitrocellulose membranes (Trans-Blot Turbo; Bio-Rad Laboratories) and blocked with 5% dried milk, and incubated in 5% BSA with primary Abs and detected with HRP-conjugated secondary Abs and ECL substrate (SuperSignal West Dura; Thermo Fisher Scientific) using a ChemiDoc Imaging System (Bio-Rad Laboratories).

Quantitative RT-PCR

Total RNA was isolated with an RNeasy Plus Kit (Qiagen) and cDNA generated with Superscript IV (Invitrogen). Quantitative real-time PCR was performed with SYBR Green PCR Mix (Applied Biosystems) using a QuantStudio 6 (Applied Biosystems). Results are relative to 18S expression. Results are expressed as the percentage of the fold increase over unstimulated divided by the fold increase over unstimulated in the absence of added corticosterone. Primer sequences used for quantitative RT-PCR are provided in Supplemental Table I.

Statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad). Unless otherwise indicated, statistical analyses were performed using an unpaired two-tailed Student *t* test. A *p* value <0.05 was considered significant. Averaged results of multiple experiments are presented as the arithmetic mean ± SEM.

Results

Effect of GCs on TCR-induced thymocyte upregulation of Nur77 and Helios

Thymocyte negative selection can be simulated in vitro by stimulating thymocytes with immobilized Abs against CD3 and CD28 (30). As reported (31), anti-CD3/CD28 stimulation increased Nur77 protein levels rapidly, peaking at 2 h and waning thereafter (Fig. 1A, 1B, left panels). Helios, also reported to be a T cell activation-induced gene (32), increased more gradually, being detectably increased by 2 h and continuing to increase through 16 h (Fig. 1A, 1B, right panels). To test whether induction of these proteins was sensitive to GCs, their levels were evaluated in anti-CD3/CD28–stimulated thymocytes in the absence or presence of corticosterone. The pan-caspase inhibitor Z-VAD-FMK was included in the cultures to prevent GC-induced apoptosis (33). Expression of Nur77 at 2 h was unaffected when corticosterone was added at the time of stimulation (Fig. 1C, left panel). When the cells were preincubated with corticosterone for 6 h, some inhibition was observed, but only at the highest concentration of corticosterone, 300 nM. Inhibition of Helios expression was also greater in cells pretreated with GCs and was much more sensitive, being detected at 30 nM corticosterone (Fig. 1C, right panel). GCs had little effect on activation-induced upregulation of CD69 (Fig. 1C, lower panels, and Supplemental Fig. 1A), and cell viability was equivalent between the groups (Supplemental Fig. 1B). Similar results were obtained with quantitative RT-PCR to measure *Nur77* and *Helios* mRNA transcripts (Fig. 1D). To evaluate whether this inhibition is at the transcriptional level, EGFP levels were examined in TCR-stimulated *Helios*/EGFP reporter thymocytes, where an IRES/EGFP cassette was inserted into the *Helios* locus (29). Consistent with regulation of transcription, *Helios* promoter-driven EGFP levels decreased in response to GCs, which was potentiated by pretreatment (Fig. 2A).

Induction of Helios is independent of Nur77

In a study profiling the induction of genes in DP cells stimulated with anti-CD3, anti-CD28, and anti-CD2, Helios was found to be upregulated less well in Nur77-deficient than wild-type (WT) thymocytes (23). This suggested that Helios was a target of Nur77, which is consistent with the timing of Nur77 and Helios upregulation (Fig. 1). However, there was no difference in Helios upregulation between WT and Nur77-deficient thymocytes, as assessed by immunoblotting (Fig. 2B, left panel) or intracellular staining and flow cytometry (Fig. 2B, right panel). We conclude that TCR-induced upregulation of Helios occurs in the absence of Nur77.

GCs inhibit activation-induced Nur77 and Helios upregulation indirectly

The inhibitory effects of GCs are often mediated by direct interference between the GR and activating transcription factors (34).

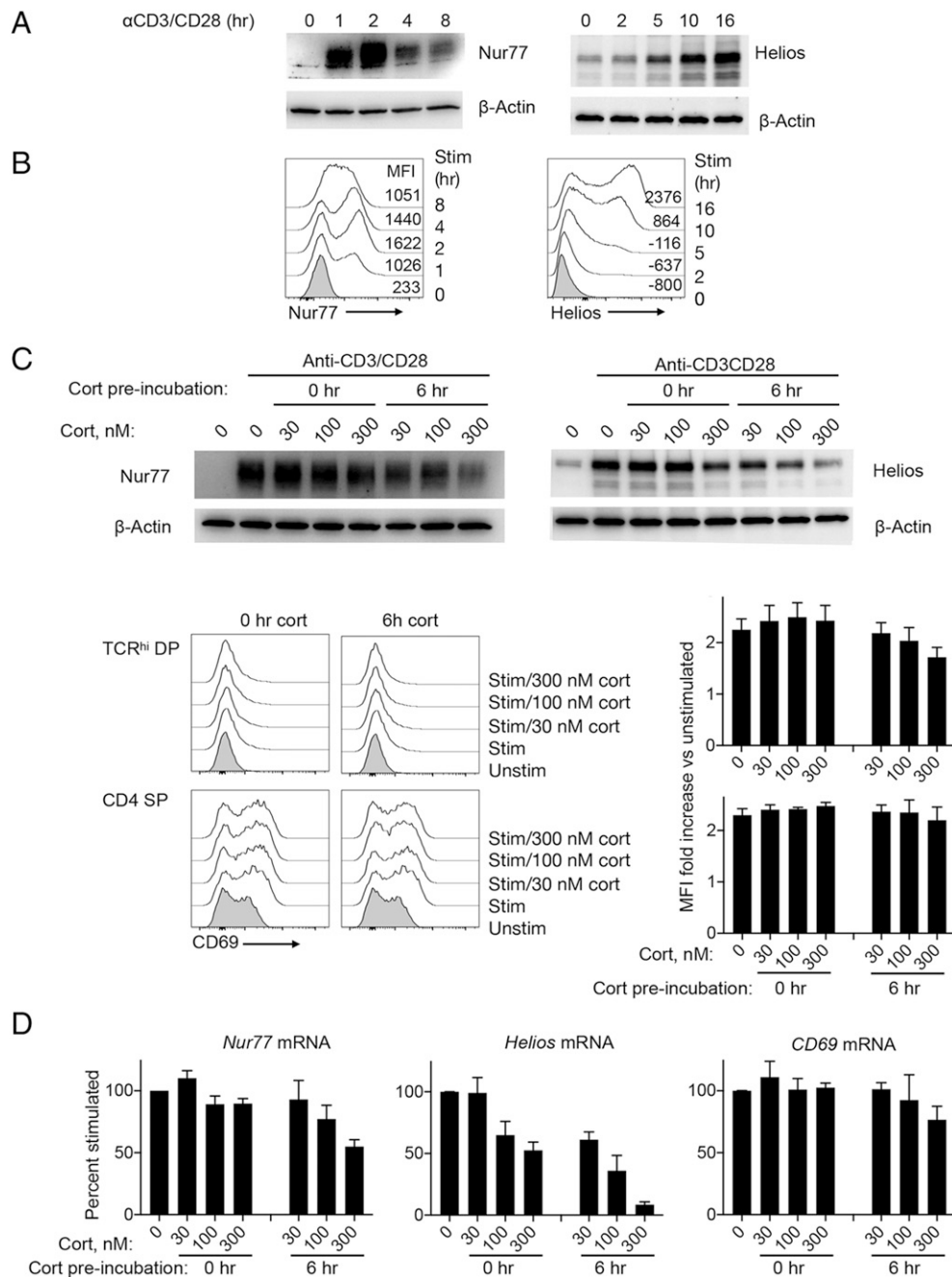
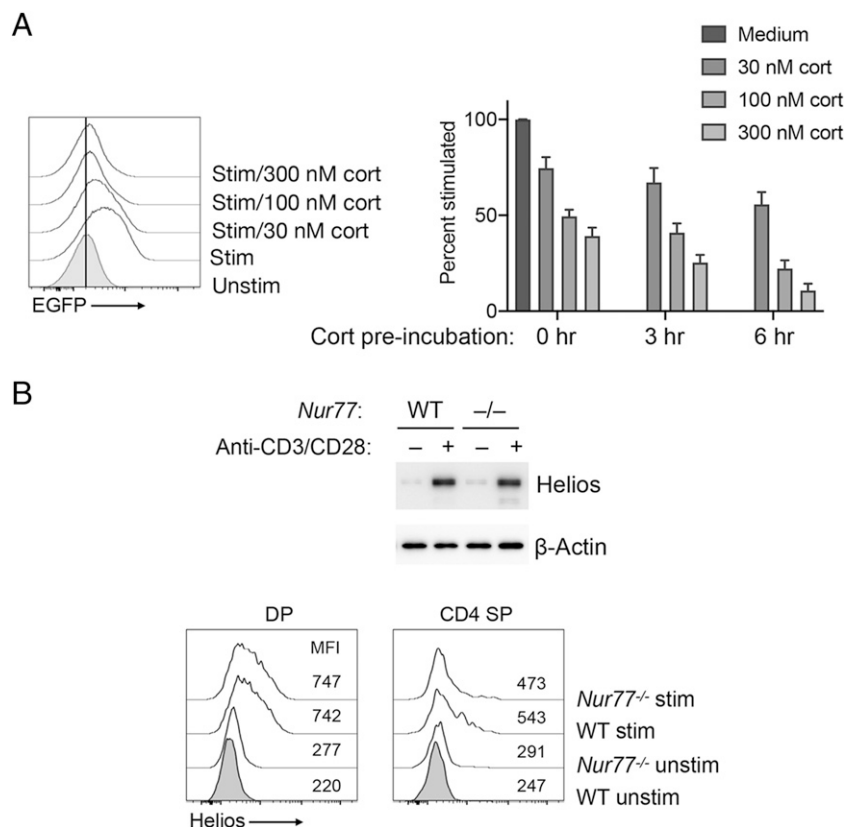


FIGURE 1. TCR-stimulated upregulation of Nur77 and Helios is sensitive to GCs. **(A)** Induction of Nur77 precedes Helios in TCR-stimulated thymocytes. Immunoblot analysis of Nur77 and Helios in whole-cell lysates from thymocytes activated for the indicated times with immobilized anti-CD3/CD28 (stimulated [Stim]). One representative blot from four experiments is shown for each protein. **(B)** Intracellular staining and flow cytometry analysis of Nur77 and Helios in thymocytes stimulated as in **(A)**. One representative plot from four experiments is shown for each protein. **(C)** Inhibition of Nur77 and Helios upregulation by corticosterone (cort). Immunoblot analysis of Nur77 and Helios from thymocytes treated with the indicated doses of cort added at the time of or 6 h prior to stimulation for 2 h (Nur77, left panel) or 10 h (Helios, right panel) by immobilized anti-CD3/CD28. Shown are representative blots and flow cytometry analysis (left) and relative mean fluorescence intensities (right) of CD69 expression on the thymocytes used for quantitation of Nur77 after 2 h of stimulation. Results are expressed as the geometric mean (95% CI). **(D)** Real-time PCR analysis of *Nur77*, *Helios*, and *CD69* mRNA from the experiments in **(C)**, expressed as the percentage of the Stim levels observed in the absence of cort. Unstim, unstimulated.

In such a case, GCs would inhibit when added at the time of, or even after, exposure to a stimulus. However, the effect of GCs on TCR stimulation-induced Nur77 and Helios upregulation increased with time of pretreatment, suggesting that it may be mediated by a GC-induced intermediary factor. To test this, the effect of corticosterone on *Nur77* and *Helios* mRNA induction was determined in the absence or presence of the protein synthesis inhibitor CHX. As shown in Fig. 3B, *Nur77* mRNA was superinduced

by CHX, a phenomenon that is due to blocking the production of RNA-degrading proteins (35). Whereas corticosterone pretreatment modestly reduced induction of *Nur77* mRNA, no reduction was seen in the presence of CHX. Although, in the case of *Helios*, there was no superinduction, but suppression of mRNA induction by GCs was similarly prevented by CHX. These results are consistent with GC-induced synthesis of one or more intermediary factors that inhibit transcription of *Nur77* and *Helios* mRNA.

FIGURE 2. (A) Inhibition of Helios upregulation by TCR stimulation in thymocytes takes place at the transcriptional level. Left panel, Flow cytometry analysis of *Helios*/EGFP reporter DP thymocytes stimulated (Stim) for 10 h with immobilized anti-CD3/CD28 plus corticosterone (cort) added 6 h before stimulation. Right panel, Mean fluorescence intensities of EGFP from *Helios* reporter DP thymocytes treated with cort for the indicated times prior to stimulation for 10 h ($n = 3$). **(B)** Helios upregulation by TCR stimulation in thymocytes is independent of Nur77. Left panel, Immunoblot analysis of lysates from and (right panel) flow cytometric analysis of Helios in WT and *Nur77*^{-/-} thymocytes Stim for 10 h with anti-CD3/CD28. Data are representative of three independent experiments with similar results. Unstim, unstimulated.



GC deficiency increases the proportion of signaled DP thymocytes undergoing negative selection

The possible roles of thymocyte Nur77 and Helios as functionally important targets of GCs was assessed by examining thymi of *AND* mice, which express an $\alpha\beta$ TCR specific for a cytochrome *C* fragment plus I-E^k MHC class II (26). Expression of this TCR in B6 (*H-2^b*) mice results in positive selection of CD4⁺ T cells (36). Thymocyte-specific deletion of the GR, achieved by crossing *GR* exon 3-floxed mice with mice expressing a *Cre* transgene driven by the *lck* proximal promoter (*GR^{lck-Cre}*) resulted in increased negative selection and a reduction in DP cell numbers (9). DP thymocytes that receive strong TCR signals upregulate their TCRs (37) as well as PD-1, Nur77, and Helios (13, 18, 38, 39). As shown in Fig. 4, GR deficiency increased the frequency of TCR^{hi} DP cells that are Nur77^{hi}/PD-1^{hi} and Helios^{hi}/PD-1^{hi} compared with GR-sufficient controls. GR deficiency had no effect on TCR or CD5 levels, consistent with no change in TCR proximal signaling but rather an effect on TCR-induced gene expression (Supplemental Fig. 2C). The small inhibition of upregulated CD69 at the highest dose of corticosterone (Fig. 1C) correlated with a small increase in in vivo CD69 levels in GR-deficient DP cells, suggesting that GCs regulate CD69 at the transcriptional level (Supplemental Fig. 2C). GR deficiency resulted in small, but not statistically significant, decreases in Treg and invariant NK T cell precursor (40) cell numbers (Supplemental Fig. 3A–C). These results demonstrate that Nur77 and Helios expression is increased in thymocytes undergoing negative rather than positive selection in Ag-signaled thymocytes in the absence of GC responsiveness.

Nur77 and Helios act at distinct stages of GC deficiency-induced negative selection

The contributions of Nur77 and Helios to enhanced deletion in GC-unresponsive thymocytes were directly assessed by examining thymi of *GR^{lck-Cre} AND* mice that were crossed with *Nur77*^{-/-} or

Helios^{fl/fl} mice. Deletion of the GR increased negative selection as shown by reduced numbers of DP and CD4⁺ SP cells (Fig. 5A, 5B). Neither the loss Nur77 nor Helios had an effect on thymocyte numbers compared with WT mice, consistent with little negative selection of the *AND* TCR transgene on the positively selecting *H-2^b* background. DP cell numbers were the same in the presence or absence of Nur77 (Fig. 5A). Surprisingly, lack of Nur77 partially restored CD4 SP numbers (Fig. 5A). The differential recovery of GR-deficient DP and CD4 SP thymocytes suggests that Nur77 may play a role in later medullary as opposed to cortical negative selection (39). In contrast to Nur77, deletion of Helios prevented GR deficiency-mediated loss of both DP and CD4 SP cells (Fig. 5B). The increased negative selection caused by GR deficiency was accompanied by increased apoptosis among signaled DP thymocytes, which was largely reversed by Helios deficiency (Fig. 5C). These results show that GCs antagonize negative selection by inhibiting TCR-signaled Helios upregulation and, perhaps to a lesser extent, that of Nur77.

Discussion

By countering TCR-mediated selection signals in thymocytes, TEC-produced GCs are critical for the generation of a robust T cell repertoire. How this happens is unknown, but given the well-established function of the GR as a transcriptional regulator, we speculated that the GR modifies thymocyte selection by regulating TCR-induced gene expression. In this report, we have identified as targets of GCs two transcriptional regulators known to be involved in negative selection and find that they play nonredundant roles in GC modulation of thymocyte selection.

Helios is a member of the Ikaros family of transcription factors and is expressed by hematopoietic stem cells during embryogenesis, where it dimerizes with Ikaros to promote B and T cell differentiation (41). Perhaps the best studied function of Helios in T lineage cells is in Tregs, where it is expressed by ~70% of the

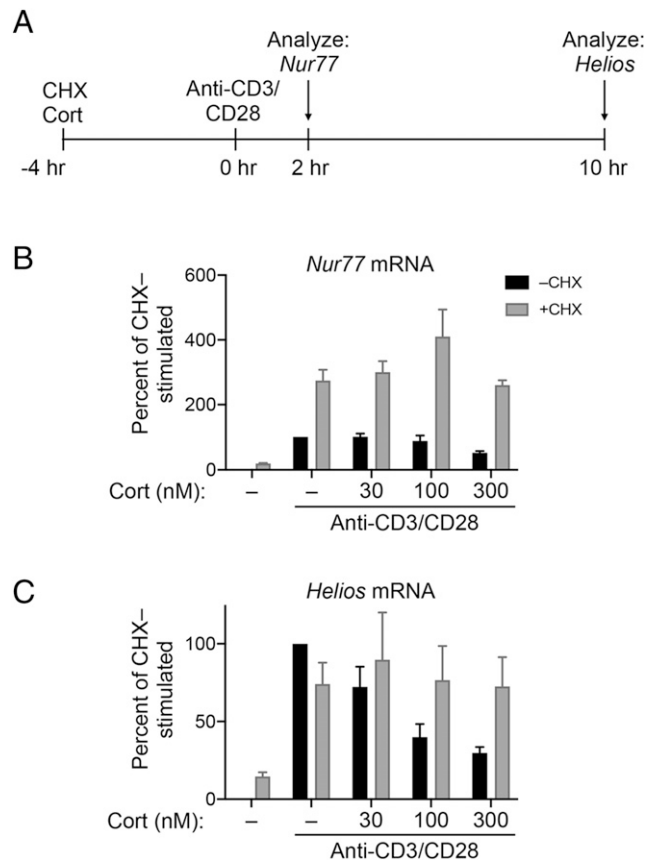


FIGURE 3. Corticosterone (cort) inhibition of *Nur77* and *Helios* mRNA requires de novo protein synthesis. **(A)** The timeline of the addition of reagents, stimulation, and analysis for the experiments shown in **(B)** and **(C)**. Quantitative real-time PCR analysis of thymocyte expression of *Nur77* **(B)** and *Helios* **(C)** mRNA treated as in **(A)**. Data are the averages of three and four independent experiments for *Nur77* and *Helios*, respectively.

cells and stabilizes Treg function (28, 42–44). *Helios* was identified as component of the nucleosome remodeling (NuRD) complex, where it may repress IL-2 production by Tregs, a key feature of their phenotype (45, 46). Although initial reports found no evidence of altered thymocyte development in *Helios*-targeted mice (28, 42, 47), the finding of elevated *Helios* levels in strongly signaled thymocytes suggested that *Helios* might contribute to

thymocyte negative selection (13, 39). Mice lacking *Helios* globally or only in Tregs were found to develop autoimmunity, but because these models were not analyzed with the same assays, the possible contribution of an altered repertoire in conventional T cells in the absence of *Helios* could not be excluded (42). Along the same lines, systemic immune activation, but not autoimmunity, was observed when *Helios* expression was disrupted in Tregs (43). A role for *Helios* in negatively selecting autoreactive TCRs has been reported in Tregs themselves. *Helios*-deficient Tregs proliferated more strongly than WT in response to activated syngeneic B cells and expressed higher levels of GITR and PD-1, markers that correlate with affinity of Treg TCRs for self (48). Enhanced self-recognition by Tregs caused by *Helios* deficiency is consistent with a similar process taking place in conventional T cells. Thornton et al. (29) found that naturally occurring *Helios*-negative and -positive Tregs have nonoverlapping TCR repertoires, a result directly attributable to altered selection. Our observation that increased *Helios* expression in the absence of GC signaling correlates with increased negative selection is consistent with *Helios* being critical to selection thresholds of self-reactivity.

Nur77 was initially reported to be a mediator of apoptosis in T cell hybridomas and strongly stimulated thymocytes (18, 19). Expression of dominant-negative isoforms rescued negative selection of TCR-transgenic models (20, 38), but deletion of *Nur77* had variable effects (23, 27, 49). As with *Helios* deficiency, *Nur77*-deficient mice develop autoimmunity. This has been attributed to a number of mechanisms, including the development of inflammatory M1 macrophages and altered metabolism of T cells (50, 51). *Nur77* deficiency increased the severity of inflammatory bowel disease and experimental autoimmune encephalomyelitis, and overexpression of *Nur77* in thymocytes reduced the severity of collagen-induced arthritis (52–54), but the possible contribution of an autoreactive T cell repertoire was not addressed. Deletion of either *Helios* or *Nur77* does not lead to obvious changes in the development of polyclonal T cells, presumably because of compensatory mechanisms related to filling thymus niches. However, both factors have been shown to play a role in the negative selection of individual TCR transgenics, and in this study we find that both factors contribute the deletion of thymocytes in the absence of GC signaling.

The effects of *Helios* and *Nur77* deletion differed with respect to the rescue of DP AND thymocytes from GR deficiency–induced negative selection. Whereas deletion of *Helios* rescued both the DP and CD4 SP subpopulations, deletion of *Nur77* had a more

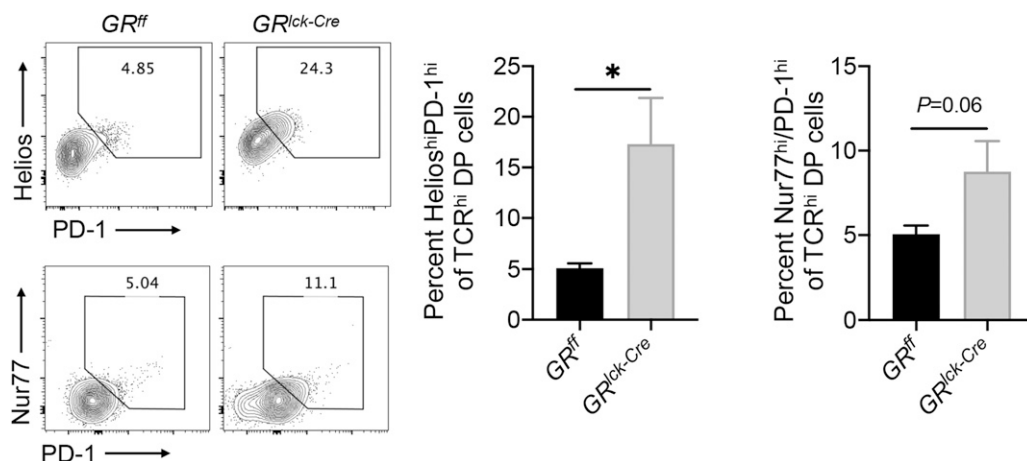


FIGURE 4. Increased *Nur77* and *Helios* expression in TCR-signaled DP thymocytes in the absence of GC signaling. Left, *Nur77*^{hi}PD-1^{hi} and *Helios*^{hi}PD-1^{hi} TCR^{hi} DP cells from GR-floxed and GR-deficient *AND TCR Rag2*^{-/-} thymocytes. Right, Means and SEM of the proportion of *Nur77*^{hi}PD-1^{hi} ($n = 8$) and *Helios*^{hi}PD-1^{hi} ($n = 16$) in TCR^{hi} DP thymocytes. Gating strategy is shown in Supplemental Fig. 2A. * $p < 0.05$.

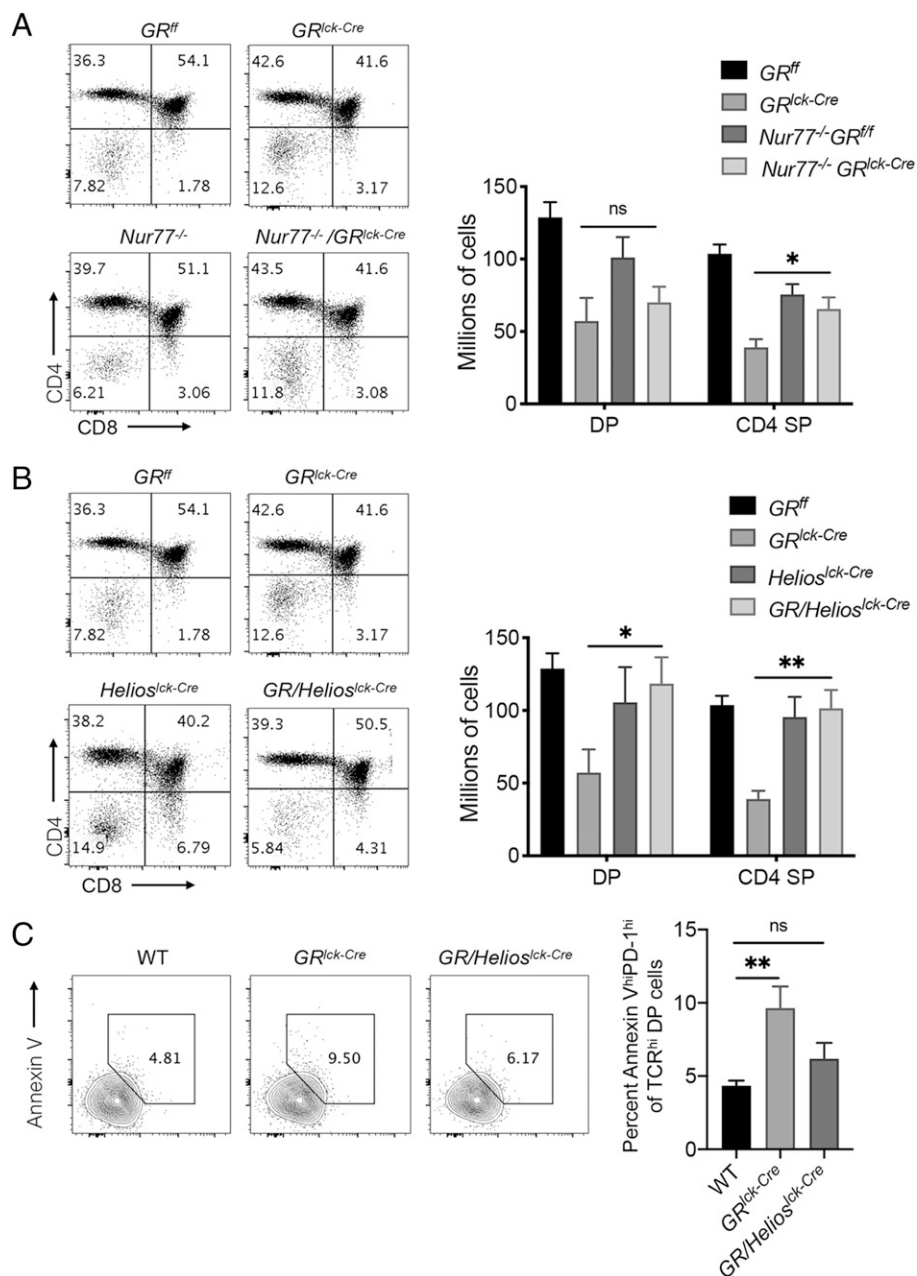


FIGURE 5. Increased negative selection of thymocytes developing in the absence of GC signaling due to GR deficiency is differentially rescued by Nur77 and Helios deficiency. Thymocyte subsets from 5-wk-old *GR^{ff}* ($n = 15$), *GR^{lck-Cre}* ($n = 5$), *Nur77^{-/-}* ($n = 6$), and *Nur77^{-/-}/GR^{lck-Cre}* ($n = 8$) AND *TCR Rag2^{-/-}* mice (A) and from 5-wk-old *Helios^{lck-Cre}* ($n = 5$) and *Helios/GR^{lck-Cre}* ($n = 5$) AND *TCR Rag2^{-/-}* (B) mice. The same cohorts of *GR^{ff}* and *GR^{lck-Cre}* thymocytes were used for (A) and (B). (C) PD-1^{hi} Annexin V⁺ DP cells (left) and frequencies (right) of *GR^{ff}* ($n = 11$), *GR^{lck-Cre}* ($n = 11$), and *Helios/GR^{lck-Cre}* ($n = 6$) AND *TCR Rag2^{-/-}* mice. Gates were selected to include the top 5% of PD-1^{hi} Annexin V⁺TCR DP cells from *GR^{ff}* mice. * $p < 0.05$, ** $p < 0.005$. ns, not significant.

complicated outcome, rescuing CD4 SP cells but having no effect on DP cells. This suggests that Nur77 plays no role in deletion by ubiquitous Ags, which are expressed in the cortex, and is consistent with the observation that Nur77 deficiency did not rescue negative selection of the AND TCR on the H-2^S MHC haplotype (27). In contrast, Nur77 has been implicated in negative selection of SP cells by medullary tissue-restricted Ags. Nur77 deficiency in OT-II and OT-I TCR transgenics rescued SP thymocytes from negative selection induced by transgenic expression of their cognate Ag, OVA (23, 24). The self-peptides that mediate positive selection of the AND TCR on the H-2^b MHC background used in this study are unknown, but it is plausible that, in addition to being expressed in the cortex, they are expressed in the medulla, where they could serve as negatively selecting ligands for SP thymocytes lacking the GR. Supporting this notion, thymi from AND mice lacking thymic production of GCs contained more apoptotic CD4 SP cells than controls (10). The levels of Helios and

Nur77 expressed in thymocyte subpopulations during TCR stimulation may be related to their function. We found Nur77 to be upregulated strongly by TCR stimulation in both DP and SP thymocytes, but its expression remained higher in SP during prolonged stimulation. In contrast, Helios levels induced during long-term stimulation were similar in DP and SP thymocytes (Supplemental Fig. 3D). Long-term stimulation of thymocytes in vitro may be relevant to the selection process, which entails prolonged contact between thymocytes and APC. Our results are consistent with Helios participating in negative selection in DPs, and perhaps SPs, whereas Nur77 facilitates negative selection of SPs that have been positively selected on cortical ubiquitous Ags. Despite being expressed in TCR-stimulated DP thymocytes, Nur77 is dispensable for negative selection of ubiquitous Ag-responsive TCR-transgenic models reported to date, including the GR-deficient AND DP thymocytes on the H-2^b background reported in this study.

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Disclosures

The authors have no financial conflicts of interest.

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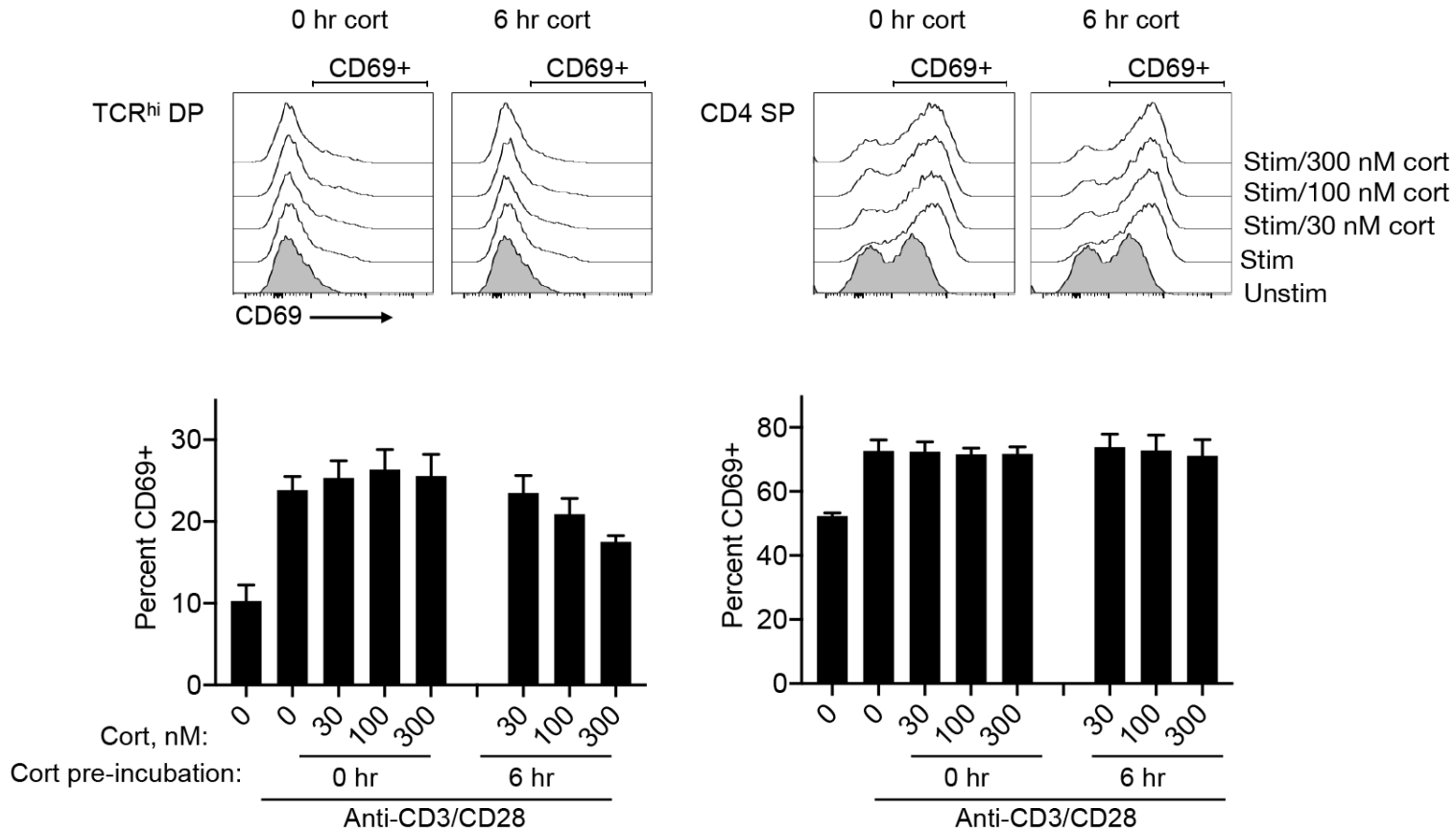
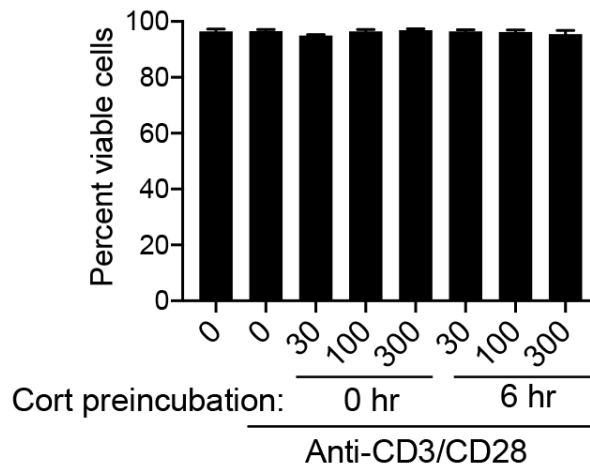
A**B**

Figure S1: (A) CD69 induction is partially sensitive to inhibition by corticosterone. CD69⁺ gates for TCR^{hi} DP (left) and CD4 SP (right) cells are shown above the flow profiles in the upper panels. Percent CD69⁺ TCR^{hi} DP (left) and CD4 SP (right) cells from the experiments in Fig. 1C are shown in the lower panels. (B) Average viability for the experiments in Fig. 1D.

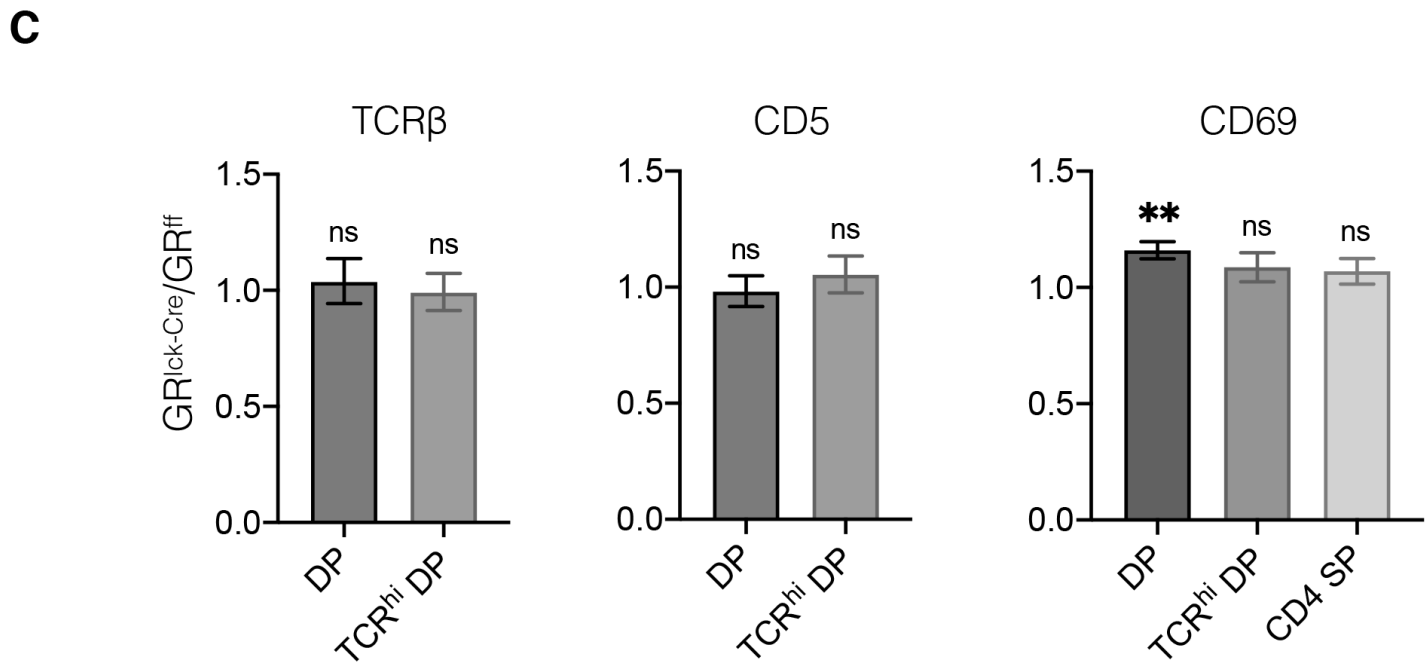
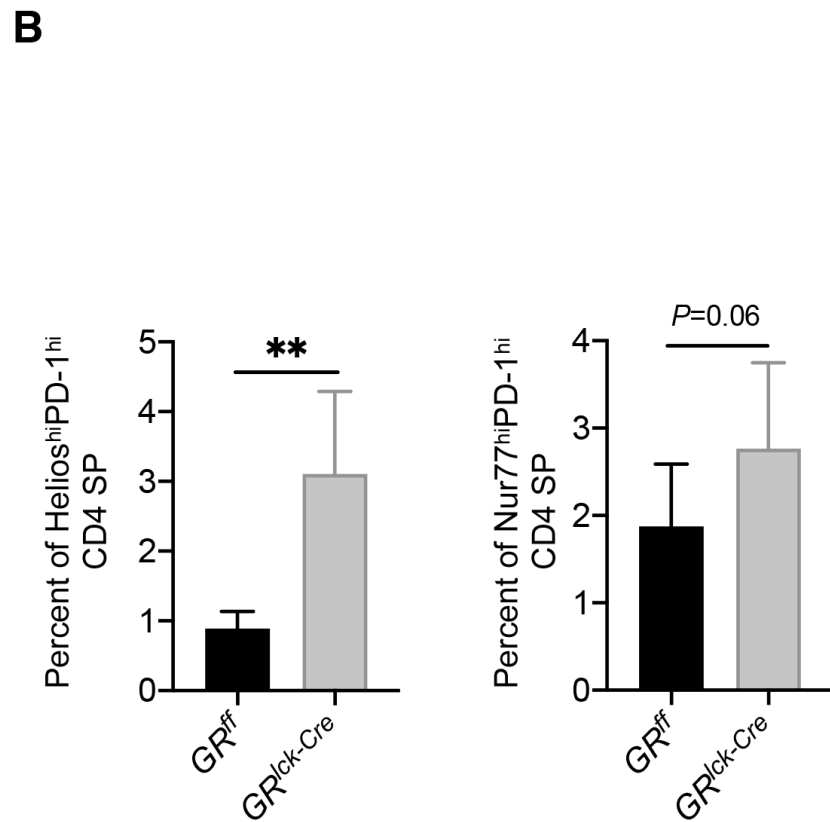
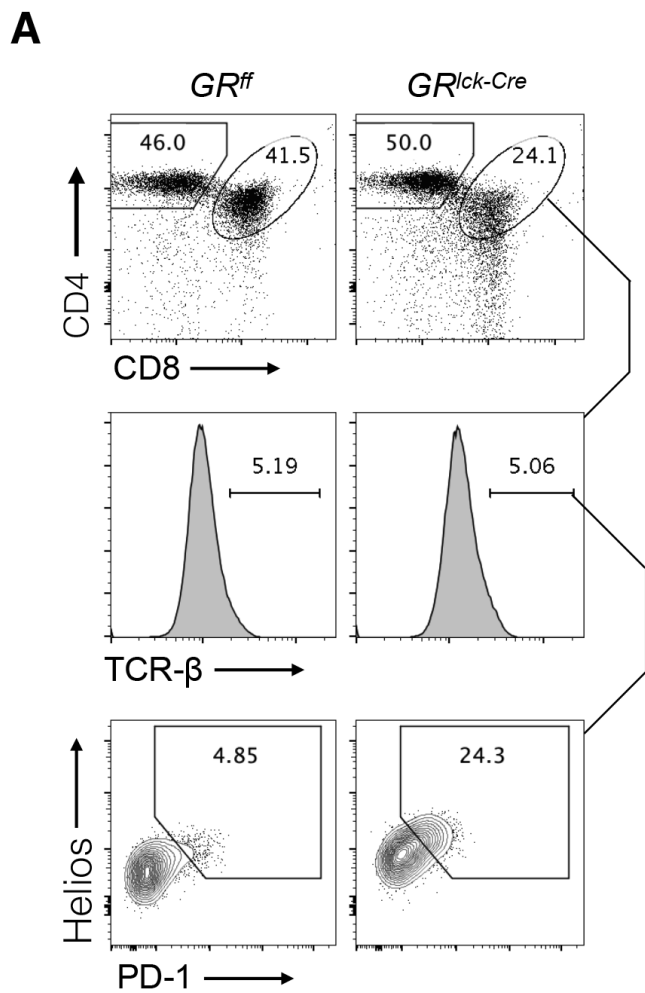


Figure S2: (A) Gating strategy for TCR^{hi} DP cells analyzed in Fig. 4. (B) Helios^{hi}PD-1^{hi}TCR^{hi} CD4 SP thymocytes from Fig. 4. (C) Effect of absence of the GR on *in vivo* TCRβ, CD5, or CD69 levels. Ratios of the MFIs of TCRβ (n= 16), CD5 (n=12), and CD69 (n=11) in the AND thymocytes. Results for (C) are expressed as the geometric mean (95% CI). ***p*<0.005. ns = not significant.

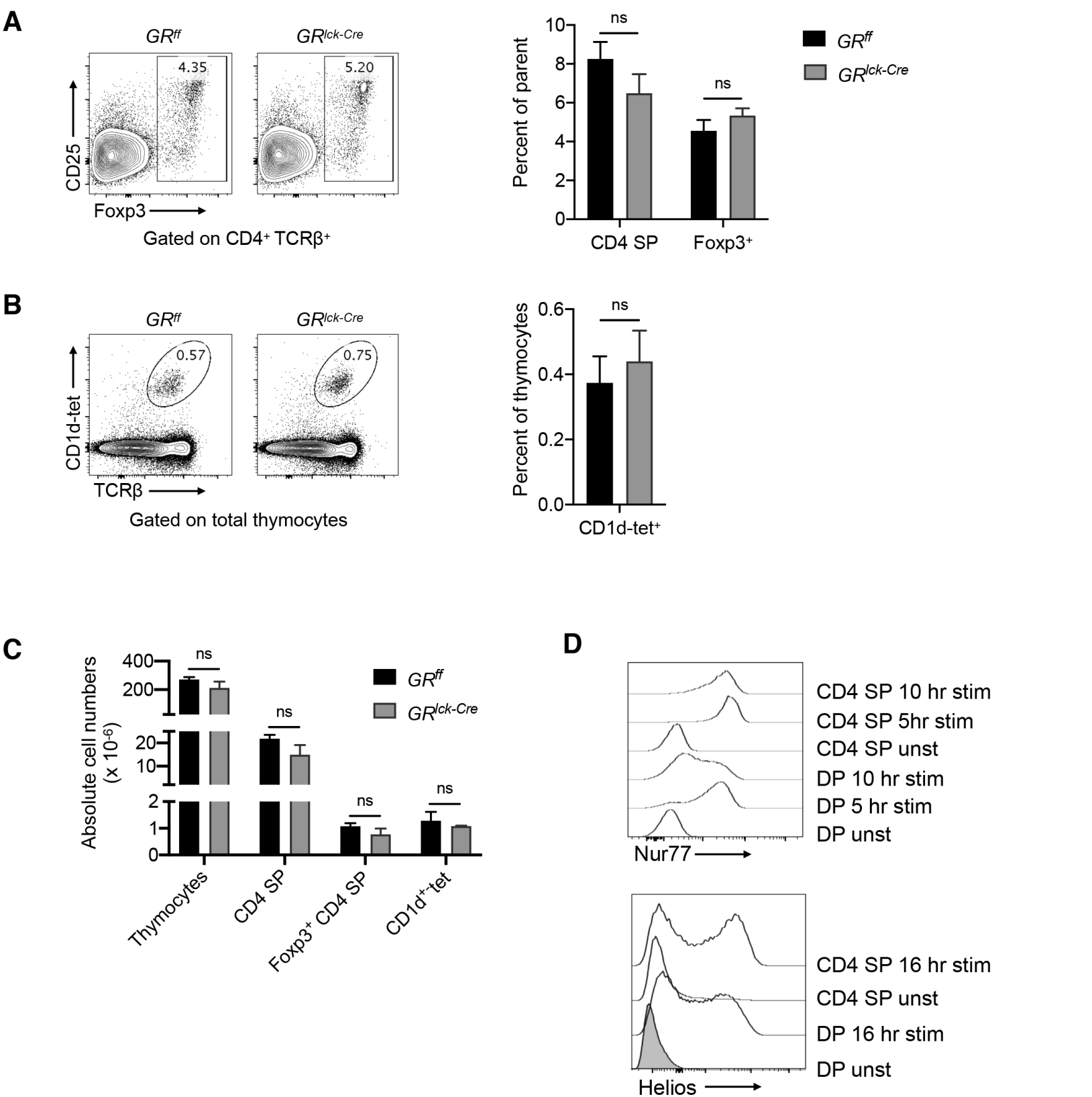


Figure S3. GR deficiency does not result in increased Treg and iNKT precursors. (A) Flow cytometric analysis (left) and frequencies (right) of Foxp3⁺ regulatory T cells in control (GR^{ff}) or GR-deficient (GR^{lck-Cre}) thymocytes. (B) Flow cytometric analysis (left) and frequencies (right) of CD1d-tetramer⁺ iNKT cells. (C) Absolute cell numbers from (A) and (B). n=5. ns = not significant. (D) Loss of Nur77 but not Helios expression in DP thymocytes during prolonged TCR stimulation. WT thymocytes were left untreated or stimulated with immobilized anti-CD3/CD28 for the indicated times and analyzed for intracellular Nur77 and Helios by flow cytometry.

Supplemental table S1

Primers

Genotyping

| | |
|---------------|--|
| GR 3' loxp | TGCCACTATACCACTGCTACTGAGAACTG TGGTTGTTGGTGCTTTTGGCTAAATC |
| Lck-Cre | CCTTGGTGGAGGAGGGTGGAAATGAA AATGTTGCTGGATAGTTTTTACTGC |
| AND TCR F | GACTTGGAGATTGCCAACCCATATCTAAGT TGAGCCGAAGGTGTAGTCGGAGTTTGCATT |
| AND ctrl F | CTAGGCCACAGAATTGAAAGATCT |
| AND ctrl R | GTAGGTGGAAATTCTAGCATCATCC |
| Nur77 WT F | CCACGTCTTCTTCCTCATCC |
| Nur77 WT R | TGAGCAGGGACTGCCATAGT |
| Nur77 KO F | TTGAGGACTTCCAGGTGTACGG |
| Nur77 KO R | CTAGTGAGACGTGCTACTTCCATTG |
| Helios flox F | CTGAGCCTCAGACAATTGGA |
| Helios flox R | TATGTGACCACACAAAGGGG |

Real-time PCR

| | |
|--------|--|
| 18S | AAATCAGTTATGGTTCCTTTGGTC GCTCTAGAATTACCACAGTTATCCAA |
| Helios | TCACTGGAGGAACACAAGGA CATAGGCGGTACATGGTGAC |
| Nur77 | TGATGTTCCCGCCTTTGC CAATGCGATTCTGCAGCTCTT |