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Thymocyte Apoptosis Induced by T Cell Activation Is Mediated by Glucocorticoids In Vivo

Judson A. Brewer,* Osami Kanagawa,† Barry P. Sleckman,‡ and Louis J. Muglia*§

Glucocorticoids, administered in pharmacological doses, potently modulate immune system function and are a mainstay therapy for many common human diseases. Physiologic production of glucocorticoids may play a role in optimization of the immune repertoire both centrally and peripherally. Possible effects include alteration of lymphocyte development and down-regulation of cytokine responses, but essential roles remain unclear. To determine the part that endogenous glucocorticoids play in thymocyte development, we used fetal liver from mice lacking the glucocorticoid receptor GRko for immunological reconstitution of lethally irradiated wild-type (WT) mice. We find normal numbers and subset distribution of GRko thymocytes. GRko thymocytes also exhibit similar sensitivity to apoptosis induced by activating anti-CD3ε Ab as WT thymocytes in vitro. Surprisingly, GRko thymocytes are significantly more resistant than WT thymocytes to anti-CD3ε-mediated thymocyte apoptosis in vivo. Consistent with this finding, in vivo TCR complex activation induces sustained high levels of glucocorticoids that correlate strongly with thymocyte apoptosis in WT mice. We find that while direct engagement of the TCR complex may cause death of a subset of thymocytes, glucocorticoids are required for deletion of the majority of thymocytes. Thus, TCR stimulation by Ab administration may more accurately reflect polyclonal T cell activation than negative selection in vivo. The Journal of Immunology, 2002, 169: 1837–1843.

Glucocorticoids, administered in pharmacological doses, are effective therapeutic agents for treatment of asthma, rheumatological disease, and hematopoietic malignancies based upon their anti-inflammatory, immunosuppressive, and cytolytic properties. Similarly, endogenous physiological production of these steroids has been suggested to play a role in optimization of the immune repertoire by affecting lymphocyte development and down-regulation of immune responses in mature T cells and macrophages (1).

Beginning with the observation over three-quarters of a century ago that removal of systemic glucocorticoids results in increased thymus size and cellularity (2), and more recently with the demonstration that TCR activation rescues T cell hybridomas and primary thymocytes from steroid-mediated apoptosis in vitro (3), glucocorticoids have been postulated to influence thymocyte development. Consistent with these observations, glucocorticoids modulate developmental pathways critical for thymocyte ontogeny, with effects on ZAP-70, linker for activation of T cells, and NF-κB signaling (1, 4, 5). However, the consequences of these interactions for the make-up of T cell repertoire remain controversial. Studies performed in fetal thymic organ culture (FTOC), using pharmacologic blockade of steroid biosynthesis, have suggested that glucocorticoids are important for thymocyte survival (6). More recent experiments have used FTOC and dispersed cell culture of thymocytes from mice in which a neomycin resistance cassette was inserted into exon 2 of the glucocorticoid receptor (GR) gene locus. Studies with this GR hypomorph allele did not find glucocorticoids essential for fetal thymocyte survival (7, 8), but were unable to assess whether the increases in thymocyte number and changes in subpopulation distribution normally found in postnatal and adult animals occurred in the absence of GR activity. Glucocorticoid contributions to thymocyte development in the adult animal have also been studied, using antisense transgenic mouse models. In this approach too, results have differed between systems, as evaluation of one model showed a decrease, while another showed an increase in thymic cellularity, with reduction of GR expression (9, 10).

In the periphery, glucocorticoids are critical for down-regulating the inflammatory response to pathogens and their toxins. Induced by cytokine stimulation of the hypothalamic-pituitary-adrenal axis, glucocorticoids potentiate the acute phase response while acting in a negative feedback loop to suppress further cytokine production (11–13). In instances of massive peripheral immune activation, such as in septic shock, endogenous glucocorticoids have been shown to be critical for the prevention of cytokine-mediated multiorgan failure and associated mortality (14). Interestingly, with polymicrobial sepsis, glucocorticoid levels sufficient to induce thymocyte apoptosis are achieved, suggesting glucocorticoid actions on the thymus may contribute to immunoregulation in instances of polyclonal or nonspecific peripheral activation (15). However, links between direct T cell activation and thymocyte killing have not been established.

In this report, we generated mice deficient in the GR and studied thymocyte development in wild-type (WT) mice reconstituted with WT and GR-deficient fetal liver cells. We found that direct GR signaling in thymocytes is not critical for normal development. Surprisingly, using a standard model of in vivo negative selection,
we showed that systemic glucocorticoids induced by TCR activation are necessary and sufficient to cause double-positive (DP) thymocyte apoptosis, suggesting that TCR activation induced by Ab administration may more accurately reflect the effects of glucocorticoids stimulated after polyclonal T cell activation than negative selection in vivo.

Materials and Methods

Animal husbandry

All mouse protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Washington University School of Medicine (St. Louis, MO). Mice were housed on a 12 h/12 h light/dark cycle with ad libitum access to rodent chow. Matings of estrous females with males were determined by detection of a copulation plug on the morning following introduction into the male cage. After detection of a copulation plug, females were removed from the male cage with the morning of plug detection designated as 0.5 days gestation to obtain accurate gestational timing. Unless otherwise noted, all mice used were 6–10 wk old and of a C57BL/6 × 129/Sv genetic background.

Generation of GRko mice

To build our GRko targeting vector (pGRloxPneo), we inserted a loxP site into the unique SacI site in the GR gene region upstream of exon 2 between exons 1B and 1C using synthetic oligonucleotides with SacI-compatible single-strand overhangs. A PGKneo cassette containing flanking loxP sites was then subcloned into an Spel restriction site in intron 2 using oligonucleotide primers. To obtain embryonic stem (ES) clones having replaced one copy of the endogenous murine GR locus with the GRloxPneo allele, TC1 ES cells underwent electroporation with linearized pGRloxPneo as we have previously described (16). DNA from 87 G418-resistant clones was subjected to Southern blot analysis using a probe external to the recombination of the targeting vector into the endogenous GR locus as indicated. ES cells heterozygous for the GRloxPneo allele containing the heterozygous GR locus with the GRloxPneo allele, TC1 ES cells underwent electroporation with linearized pGRloxPneo as we have previously described (16). DNA from 87 G418-resistant clones was subjected to Southern blot analysis using a probe external to the flanking regions within our targeting vector. Five clones demonstrated homologous recombination of the targeting vector into the endogenous GR locus as evidenced by the appearance of a 4-kb restriction fragment length polymorphism. ES cells heterozygous for the GRloxPneo allele containing the distal loxP site were transiently transfected with the Cre-expression vector pMC Cre. DNA isolated from colonies of individually plated cells following transfection was analyzed by Southern blot. Clones having deleted both exon 2 and the neocassette were identified (GRko allele). One clone heterozygous for the knockout allele was injected into C57BL/6 blastocysts and resulted in germline transmission of the ES genome.

Fetal liver reconstitution

Female C57BL/6 mice were lethally irradiated (900 rad) and reconstituted with fetal liver cells from heterozygous GRko matings as previously described (17). Briefly, embryos were harvested 14.5–16.5 days postcoitus, and fetal livers were dispersed in DMEM using a 20-gauge needle. Embryos were genotyped by PCR. Experiments were performed 6 wk after reconstitution. Reconstitution was confirmed by FACS analysis of thymocytes for surface expression of Ly9.1 (99% of cells were Ly9.1+ in representative WT and GRko reconstituted mice).

Corticosterone assay

Plasma concentration of corticosterone was determined by RIA (ICN Pharmaceuticals, Costa Mesa, CA) from blood collected by retroorbital phlebotomy at cardiac nadir in singly housed adult male mice as previously described (18).

In vitro thymocyte apoptosis

Whole thymocytes (5 × 10^6) were cultured in 0.2 ml of RPMI supplemented with 5 × 10^-3 M 2-ME, l-glutamine, and 10% heat-inactivated FCS in 96-well tissue culture plates coated with monoclonal anti-CD3ε Ab (145-2C11) at the indicated concentrations of corticosterone (Sigma-Aldrich, St. Louis, MO) for 20 h. After harvesting, cells were washed and analyzed for apoptosis using an annexin-V apoptosis detection kit (BD Pharmingen, San Diego, CA) on a FACScalibur (BD Biosciences, Mountain View, CA). Percentage of specific killing was calculated using the following formula: (experimental apoptosis – spontaneous apoptosis)/ (100 – spontaneous apoptosis).

In vivo treatment for deletion of thymocytes

Mice were injected i.p. with 50 μg monocular anti-CD3ε Ab (145-2C11, prepared from mouse ascites, endotoxin level: 59 pg/mg), 50 μg anti-TCRβ Ab (H57-597), 50 μg isotype control Ab (both from BD Pharmingen, endotoxin level: ≤10 ng/ml) diluted in 200 μl PBS, 200 μg dexamethasone (DEX) phosphate, or normal saline, using a 50-gauge needle. Thymocytes were harvested 24 (DEX) or 48 (Ab) h after injection, dispersed through nylon mesh into PBS, washed, counted on a hemocytometer, stained for cell surface markers (FITC-anti-CD25, PerCP-anti-CD4, PE-anti-CD8, FITC-anti-CD69, and PE-anti-TCRβ from BD Pharmingen), and analyzed by FACS. Nonsciable cells were gated out based on forward and side scatter profile.

Western blot analysis

Total liver protein from newborn mice was hybridized with amino terminus (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) or DNA-binding domain (BaxGR2; Affinity BioReagents, Golden, CO) Abs at a 1/200 dilution and developed using HRP-conjugated anti-rabbit antiserum at a 1/1000 dilution using ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then stained with Ponceau S solution (Sigma-Aldrich) to ensure equal loading of protein.

Histology

Lungs from newborn mice were isolated and fixed in 4% paraformaldehyde in PBS, embedded in paraffin, cut into 5-μm sections, and stained with H&E.

Statistical methods

Results are expressed as mean ± SEM unless otherwise indicated. Statistical analysis was done by ANOVA with p < 0.05 considered significant.

Results

GR deficiency results in abnormal lung development and perinatal mortality

To functionally inactivate the GR, we generated mice which harbor a deletion of both exons 1C and 2 of the GR gene (GRko; Fig. 1A). Heterozygous GRko mice appeared grossly normal and were fertile. However, in >100 progeny arising from GRko heterozygous × GRko heterozygous matings that were genotyped at 3 wk of age, none were homozygous GRko. To determine whether homozygous GRko mice die in utero or at birth, we evaluated timed pregnancies from GRko heterozygous matings. We noted litters of normal size, but 5/22 (23%) of the resulting offspring died within 24 h of birth. When genotyped, these all proved to be homozygous GRko.

To measure the degree of GR deficiency our deletion imparted in GRko mice, we performed Western blot analysis of newborn mouse tissues, using Abs specific for the amino terminal and more C-terminal DNA-binding domains of GR. We found reduced GR expression in heterozygous mice and a complete absence of the GR protein in GRko mice (Fig. 1C). As glucocorticoids have been shown to be important in pulmonary maturation (19, 20), we examined the lungs of newborn mice. Consistent with previous reports, histologic analysis of GRko mice revealed thickened alveolar septae, poor air space formation, and hypercellularity compared with WT littermates confirming the GR functional deficiency in these mice (Fig. 1D).

GR-deficient thymocytes develop normally in vivo

Glucocorticoids have been postulated to be important for the development of normal thymocyte numbers and subpopulations (6, 9). However, in the embryo, glucocorticoids have also been reported to be dispensable for normal development (8). To determine the role of GR signaling in utero, we analyzed thymocyte numbers and subpopulations at embryonic day 16.5, at which time CD4/CD8 DP thymocytes begin to accumulate in the thymus. Consistent with previous reports (8), we noted no difference in thymocyte...
numbers and subpopulation percentages between GR-deficient and GR-intact embryos (total thymocytes (× 10⁶): GRko = 1.33 ± 0.22, WT/GR heterozygous (GRhet) = 1.4 ± 0.48; CD4/CD8⁺ (%): GRko = 28.0 ± 4, WT/GRhet = 32.5 ± 4, n = 2 and 4, respectively). Additionally, thymocyte numbers and subpopulations in GR-deficient mice were no different from their GR-intact counterparts at birth (total thymocytes (× 10⁶): GRko = 13.6 ± 3.4, WT/GRhet = 11.0 ± 4.4; CD4/CD8⁺ (%): GRko = 80.0 ± 4.2, WT/GRhet = 78.1 ± 7.0; CD4⁺ (%) GRko = 3.7 ± 0.78, WT/GRhet = 3.7 ± 1.1; CD8⁺ (%): GRko = 3.2 ± 0.49, WT/GRhet = 3.2 ± 1.0; n = 2 and 7, respectively). These data suggest no requirement for GR in embryonic thymocyte development.

To determine the role of glucocorticoids in adult thymocyte development in vivo, we reconstituted WT mice with hematopoietic stem cells from GRko fetal liver. Thymocyte numbers and CD4/CD8 subpopulations in reconstituted WT, heterozygous, and GRko mice did not differ (Fig. 2). Additionally, TCRβ and CD69 expression in GRko thymocytes was no different from WT thymocytes (data not shown). These data suggest that thymocyte GR activation is not required for expansion and differentiation to DP and single-positive cells in vivo.

GR-deficient thymocytes resist DEX-induced apoptosis in vivo
Glucocorticoids reliably induce substantial DP thymocyte apoptosis (21). To confirm that GRko thymocytes resist glucocorticoid killing in vivo, we reconstituted lethally irradiated WT mice with GRko fetal liver cells and assessed thymocyte numbers and subpopulations after administration of DEX, a selective GR agonist. GRko thymocytes were resistant to DEX, in contrast to heterozygous and WT mice, which showed a large reduction in DP populations (Fig. 2).

Glucocorticoids kill thymocytes more efficiently than TCR stimulation in vitro
Because mice with GR-deficient thymocytes have no reduction in total thymus cellularity, suggesting that GR is dispensable for normal expansion, we next tested whether GRko thymocytes could undergo efficient deletion with TCR activation in vitro. As negative selection of DP thymocytes can be mimicked using anti-TCR stimulation, we cultured reconstituted thymocytes with increasing concentrations of anti-CD3e Ab. Both WT and GRko thymocytes died in a similar dose-dependent manner with Ab activation (Fig. 2).
presence of anti-CD3 inflammatory response, in the levels to those achieved during an in mouse glucocorticoid, corticosterone, ranging from basal mocytes in vitro with physiologic concentrations of the endoge-plex activation to that of glucocorticoids, we incubated WT thy-mocytes. Surprisingly, GRko thymocytes were markedly resistant to administration killed survival and distribution 48 h later. As expected, we found that Ab injection with CD3 cross-linking in (22). We observed a large rise in plasma corticosterone after Ab injection, although of a lower magnitude than after anti-CD3Ab administration (Fig. 5A). Again, this corticosterone rise directly correlated (p < 0.01 at both 24 and 48 h) with a significant, but less pronounced reduction in DP thymocytes (isotype control (×10^6) = 139.1 ± 17.4, H57-597 = 74.1 ± 15.9, n = 4–5, p = 0.02).

Taken together, these in vitro and in vivo data suggest that anti-TCR Ab administration elicits a strong and prolonged peripheral glucocorticoid surge, which is necessary and sufficient to cause massive DP thymocyte apoptosis.

Glucocorticoids mediate thymocyte apoptosis from TCR complex activation in vivo

As GRko thymocytes die normally with activation in vitro, we sought to determine whether this was also true in vivo. Using a standard model of in vivo negative selection, we administered anti-CD3 Ab to fetal liver-reconstituted mice and measured thymocyte survival and distribution 48 h later. As expected, we found that Ab administration killed ~90% of WT and heterozygous DP thymocytes. Surprisingly, GRko thymocytes were markedly resistant to anti-CD3-induced apoptosis (Fig. 4). Because GRko thymocytes do not show a defect in killing caused by CD3 cross-linking in vitro, these results suggest that GR function is not required for apoptosis induced by TCR complex activation intrathymically. Instead, our results suggest that mature T cell activation may be causing a abnormal and sustained peripheral glucocorticoid surge sufficient for killing thymocytes in a process that does not reflect normal negative selection.

To test the hypothesis that mature T cell activation was causing a peripheral glucocorticoid surge capable of inducing thymocyte apoptosis, we measured plasma corticosterone concentrations in WT mice at various times after anti-CD3 Ab administration. Corticosterone levels shown to kill 90% of thymocytes in vitro were achieved by 24 h and maintained at 48 h after Ab administration (Fig. 5A). Additionally, blood corticosterone levels at both 24 and 48 h correlated strongly with thymocyte apoptosis (Fig. 5B).
GRko fetal liver were injected with 50 μg glucocorticoids in vivo. Mice reconstituted with WT, heterozygous, or sense GR-decient fetal liver, we noted the normal distribution of thymic subpopulations. We did not observe a requirement for GR for normal expansion as previously reported in one line of transgenic sense GR mice (9).

Although our studies show that thymocyte development is grossly normal in the absence of GR action, they do not prove that mature T cells with normal peptide/MHC avidity are exiting the thymus and generating an unaltered peripheral T cell repertoire. In fact, several lines of evidence suggest that glucocorticoids may be affecting lymphocyte repertoire in ways that our experimental paradigm cannot examine. First, adrenalectomy increases both thymus weight and cellularity (2). Second, corticotropin releasing hormone-deficient animals that lack circadian glucocorticoid surges have a 50% increase in thymus size (23). These observations suggest that systemic glucocorticoids may facilitate clearance of cells destined to die by neglect or negative selective processes. Additionally it is possible, as with any knockout system, that as yet unidentified GR-like receptors are compensating for GR signaling to provide grossly normal thymocyte development.

Because the demonstration that survival of DP thymocytes was sensitive to administration of anti-CD3ε Abs, Ab-induced TCR activation has become a widely used model of thymocyte negative selection in vivo (24). The observations that Ab administration induces CD4/8 expression and thymocyte expansion in RAG2−/− mice (25) and reduced surface expression of CD25 on CD4/8− cells in our mice (anti-CD3ε Ab- and saline-treated mice had 7 ± 1 and 43 ± 2% CD25+/CD4−/CD8− cells, respectively), suggest that Ab injected i.p. directly effects the thymus. Based upon the slight reduction in cell number in our GRko reconstituted mice, direct action of anti-CD3ε may be causing modest DP thymocyte apoptosis in vivo. However, unlike negative selection experiments in vitro, where the extracellular milieu is well controlled, cytokines...
and/or hormones elicited by mature T cell activation may also be involved in inducing thymocyte apoptosis in vivo (22). Indeed, we find robust induction of CD69 and IL-2 production in both WT and GR-deficient splenic T cells exposed to anti-CD3ε (J. A. Brewer and L. J. Muglia, unpublished observations). We and others have shown that administration of CD3ε-activating Ab induces many cytokines, several of which secondarily stimulate high systemic glucocorticoid levels (18, 26). Are these levels sufficient to kill DP cells? Indeed, sustained corticosterone levels of as little as 100 ng/ml have been shown to delete a majority of thymocytes in vivo (27). Additional studies in which WT mice were adrenalectomized and replaced with normal basal plasma levels of corticosterone (5 or 50 ng/ml) further support the necessity of a glucocorticoid surge to cause thymocyte apoptosis after anti-CD3ε Ab administration (L. J. Muglia, unpublished observations). Because of very high mortality in mice with plasma corticosterone clamped at basal levels after 12 h of anti-CD3ε Ab exposure, reduction in thymocyte number could not be assessed. However, analysis of DNA ladder- as evidence for early apoptosis revealed robust DNA fragmentation in sham-adrenalectomized mice that manifest an elevation of plasma glucocorticoids, while no such fragmentation was observed in those mice unable to mount a glucocorticoid response above basal levels. Linking these observations, we show that anti-CD3ε, as well as anti-TCR/β Ab administration, elicits a glucocorticoid response that far exceeds the threshold necessary for immature thymocyte apoptosis. As the daily circadian peak in circulating glucocorticoid concentration approximates the peak level achieved after CD3ε activation, but does not cause profound reduction in thymocyte number, the overall duration of glucocorticoid exposure is likely to be a key component in causing thymocyte death.

Our findings may also apply to other in vivo models of negative selection where the potential for polyclonal T cell activation is high. Relatively specific deletion of immature and semimature thymocytes occurs with oligoclonal activation by small doses of staphylococcal enterotoxin B (SEB) in WT mice where peripheral T cell contributions are minimal (28). However, SEB has also been shown to activate the hypothalamic-pituitary-adrenal axis in high doses, resulting in rapid corticosterone production to levels capable of depleting DP thymocytes (29). These observations suggest that once a threshold of T cell activation capable of mediating a systemic inflammatory response is achieved, thymocyte killing ceases to be specific, due to the elicitation of systemic glucocorticoids, and no longer models negative selection processes. Past and future studies using anti-TCR Abs, SEB, or other molecules capable of interacting with thymocytes but also activating peripheral T cell responses must be interpreted in this fashion.

Why would the organism have evolved the capacity for the immune system to promote sustained steroid production of a magnitude sufficient to kill the vast majority of immature thymocytes? We postulate that restricted oligoclonal immune responses resulting in limited cytokine production, as exemplified by viral infection, would result only in the low level of adrenal activation needed to dampen a pathogen-specific response. In contrast, nonrestricted polyclonal TCR engagement, as seen in toxic shock, may require a second line of immunomodulation to promote survival of the animal. In this setting, not only down-regulation of activated peripheral T cells, but also the blockade of naïve lymphocytes, preparing to potentiate and perpetuate uncontrolled inflammatory responses, may be required. Here, apoptosis of DP thymocytes before they mature and emigrate to the periphery, may be critical for removing potential sources of further cytokine production, thus bringing states of immune-mediated shock under control. This two-tiered mechanism of regulation, mediated by glucocorticoids, seems ideally suited to matching requirements of immunosuppression to magnitudes of inflammatory challenge.

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