Requirement for Ca$^{2+}$/Calmodulin-Dependent Kinase Type IV/Gr2+ Requirement for Ca$^{2+}$

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Requirement for Ca$^{2+}$/Calmodulin-Dependent Kinase Type IV/Gr in Setting the Thymocyte Selection Threshold

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The outcome of thymocyte selection is influenced by the nature of Ca$^{2+}$ signals transduced by the TCR. Robust Ca$^{2+}$ responses characterize high-affinity, negatively selecting peptide/TCR interactions, while modest responses typify lower-affinity, positively selecting interactions. To elucidate mechanisms by which thymocytes decode distinct Ca$^{2+}$ signals, we examined selection events in mice lacking Ca$^{2+}$/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr), which is enriched in thymocytes. CaMKIV/Gr-deficient thymocytes exhibited impaired positive selection and defective Ca$^{2+}$-dependent gene transcription. Significantly, CaMKIV/Gr deficiency raised the selection threshold of peptide/TCR interactions such that a peptide that normally induced weak negative selection instead promoted positive selection. These results demonstrate an important role for CaMKIV/Gr in sensitizing thymocytes to selection by low-affinity peptides.


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3A9 TCR-transgenic mice express a receptor specific for a hen egg-white lysozyme (HEL) peptide, amino acids 46–61, presented by the MHC class II molecule I-^A^ (30). The H-Y TCR-transgenic mice express a receptor that recognizes a male-specific Ag presented by the MHC class I molecule H-^2^ (31). Transgenic mice expressing a membrane form of HEL (mHEL) containing Hb^6^ (64–76) as an epitope tag were derived as previously described (4). The mHEL transgene is controlled by the MHC-Exa promoter, limiting expression to all class II-positive cells (4). Transgenic mice for the indicated time periods with 10^6 fibroblasts (TX). The membranes were reprobed for GAPDH transcripts to confirm equal loading.

**Antibodies**

Fluorochrome-conjugated or biotinylated mAbs directed against the following murine Ags were obtained from BD PharMingen (San Diego, CA): TCR a/b/CD3e/CD4/CD5, CD5a, CD69, and CD20. 3.12, 3.9, and H-Y TCR clonotypic mAbs were generated and used as described (29, 31, 32). Secondary streptavidin-fluorochrome conjugates were from Caltag Laboratories (Burlingame, CA). Rabbit polyclonal anti-extracellular signal-regulated kinase (ERK)1,2 Abs and mouse anti-phospho-ERK mAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-CREB Abs were from Upstate Biotechnology (Lake Placid, NY) and rabbit polyclonal anti-CREB Abs were from Cell Signaling Technology (Beverly, MA).

**Flow cytometry**

Single cell suspensions of thymocytes or splenocytes were stained in FACs buffer (PBS supplemented with 0.5% BSA and 0.1% sodium azide) using the following protocol. Aliquots of cells (10^6/sample in 100 μl of FACs buffer) were placed in polypropylene culture tubes (12 × 75 mm) and incubated on ice for 1 h with the biotinylated or directly labeled Abs. For biotinylated Abs, cells were then washed once with 3 ml of FACs buffer and incubated for 30 min on ice with streptavidin-fluorochrome conjugates (Tricolor-streptavidin or PE-streptavidin; Caltag Laboratories), as appropriate. Cells were washed again, fixed for 18–24 h in FACs buffer plus 1% paraformaldehyde, and analyzed on a FACScan (BD Biosciences, Mountain View, CA) flow cytometer using CellQuest (BD Biosciences) software. Samples were gated on live cells and 10^5 live cell events per sample were collected.

**T cell proliferation assays**

Cross-linking of mAb to tissue culture plates was achieved by first coating the plates overnight with polyclonal goat anti-hamster Abs (The Jackson Laboratory, Bar Harbor, ME) at 10^9/ml. After washing, the plates were incubated with anti-TCR mAb (Fig. 2b). Positive selection of 3.L2tg thymocytes on endogenous peptides was not affected (Fig. 1c). These results are consistent with decreased production in CaMKIV/Gr KO mice of SP thymocytes.

**Northern blot analysis**

Total RNA was derived from freshly isolated thymocytes that were either left untreated or treated with ionomycin (1 μM) and PMA (20 ng/ml) for 1 or 3 h. RNA (10 μg/lane) was loaded on a formaldehyde gel, resolved by electrophoresis, and transferred to Hybond-XL membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were hybridized with the indicated probes overnight at 42°C in ULTRAhyb solution (Ambion, Austin, TX). The membranes were reprobed for GAPDH transcripts to confirm equal loading.

**Immunoblotting**

For evaluation of phospho-CREB and phospho-ERK induction following TCR signaling, thymocytes of WT, Het, and KO littermate mice were suspended at 10^7 cells/ml in RPMI 1640 medium at 37°C and stimulated for the indicated time periods with 10 μg/ml of an anti-CD3 mAb (mAb 145-2C11; BD Pharmingen) together with 10 μg/ml of a secondary cross-linking Ab. For immunoblotting, whole cell lysates (10^6 cells/sample) were resolved by SDS-PAGE, then transferred to nitrocellulose and probed with one or more of the following Abs, as indicated: mouse anti-CaMKIV/Gr catalytic domain mAb, rabbit polyclonal anti-ERK and anti-phospho-ERK Abs, and anti-CREB and phospho-CREB Abs. The blots were developed using HRP-conjugated secondary Abs and enzyme-linked chemiluminescence (Amersham Pharmacia Biotech).

**Results**

**Impaired thymocyte maturation in CaMKIV/Gr-deficient mice**

The role of CaMKIV/Gr in T cell development was addressed using CaMKIV/Gr-deficient mice derived by targeted gene inactivation (28). Homozygous-deficient (KO) mice lacked CaMKIV/Gr expression in thymocytes and in peripheral T cells, while Het mice expressed CaMKIV/Gr at 50% of WT levels (Fig. 1a). Thymic cellularity of CaMKIV/Gr KO mice was not statistically different from that of WT littermate controls. However, flow cytometric analysis revealed a reduction in the percentages of CD4 and CD8 single-positive (SP) thymocytes in CaMKIV/Gr KO mice when compared with WT littermates (mean reduction 34% and 35%, respectively; n = 12 pairs of WT and KO mice, p < 0.001) (Fig. 1b). There was a concomitant increase in the CD4^-CD8^- (DP) compartment. Examination of TCR expression on CD4-SP thymocytes revealed decreased numbers of cells expressing high levels of TCR in KO mice compared with WT littermates. However, the level of TCR expression on KO SP cells was not affected (Fig. 1b). We also analyzed the expression in KO thymocytes of developmentally regulated Ags such as CD5 and CD69, whose levels are up-regulated in the course of positive selection. The percentage of CD5^- and CD69^- cells was decreased in KO mice to an extent commensurate with the decrease in SP thymocytes. However, the level of expression of both markers was not affected (Fig. 1c). These results are consistent with decreased production in CaMKIV/Gr KO mice of SP thymocytes.

Examination of the peripheral T cell compartment revealed a modest increase in spleen size of CaMKIV/Gr KO mice relative to WT controls. The percentages of CD4^- and CD8^- splenic T cells were mildly decreased in KO mice relative to WT controls (Fig. 2a). However, when corrected for splenic cellularity, the absolute numbers of CD4^- and CD8^- T cells were maintained. In contrast, the B cells were increased in KO mice. Both WT and KO splenocytes proliferated equally well upon stimulation with cross-linked anti-TCR mAb (Fig. 2b).

**Positive and negative selection in TCR-transgenic × CaMKIV/Gr KO mice**

The influence of CaMKIV/Gr deficiency on T cell selection events was further examined using transgenic mice expressing TCR with defined specificity. The 3.L2tg mice express T cells specific for the minor d allele of the β-chain of murine hemoglobin, amino acids 64–76, in the context of the MHC class II molecule I-^E^ (29). Positive selection of 3.L2tg thymocytes on endogenous peptides plus I-^E^ results in mature CD4^- T cells that express high levels of the transgenic TCR. Analysis of 3.L2tg × CaMKIV/Gr KO mice revealed that while the thymus size was not significantly different from WT controls, the percentage of CD4-SP thymocytes was decreased by 45% in KO mice (n = 10 pairs of WT and KO mice, p < 0.001) (Fig. 3a). There was a corresponding increase in the CD4^-CD8^- (DP) compartment. Staining with a 3.L2 TCR clonotypic mAb showed marked reduction (57%) in the generation of 3.L2tg TCR^high^ CD4-SP thymocytes in 3.L2tg × CaMKIV/Gr KO mice, consistent with the decreased generation of CD4-SP thymocytes.
The impact of CaMKIV/Gr deficiency on positive selection was further examined using the 3A9 TCR-transgenic mice, which express T cells specific for HEL (46–61) in the context of I-A<sup>k</sup> (30). Fig. 3b demonstrates that the percentage of CD4-SP thymocytes was decreased by about one-third in 3A9<sup>CaMKIV/Gr KO</sup> mice relative to 3A9 WT controls. Staining with a 3A9 TCR clonotypic mAb revealed a corresponding reduction in the generation of 3A9 TCR<sup>high</sup> CD4-SP thymocytes in 3A9<sup>CaMKIV/Gr KO</sup> mice. Together, the results obtained in the 3.L2tg and 3A9 TCR-transgenic mice confirmed that CaMKIV/Gr deficiency impairs positive selection of MHC class II-restricted, TCR-transgenic thymocytes.

The impact of CaMKIV/Gr deficiency on MHC class I-restricted positive selection was examined using TCR-transgenic mice expressing the H-Y TCR, which is specific for the H-Y male Ag in the context of H-2<sup>b</sup> (31). Thymocytes expressing the H-Y-specific TCR are positively selected along the CD8 lineage in female mice, but are deleted in male mice. Analysis of H-Y TCR<sup>H</sup> female transgenic mice demonstrated that the percentage of CD8-SP thymocytes was decreased in CaMKIV/Gr KO mice by ~50% as compared with WT mice (Fig. 4). Staining with a H-Y TCR clonotypic mAb also showed a corresponding reduction in the generation of H-Y TCR<sup>high</sup> CD8-SP thymocytes in H-Y TCR-transgenic × CaMKIV/Gr KO mice, consistent with the decreased generation of CD8-SP thymocytes (Fig. 4). The percentage of CD4-SP cells was also decreased in H-Y TCR<sup>−</sup> KO mice, while the percentage of DP cell thymocytes was increased. These results are
consistent with a block in DP to SP transition in CaMKIV/Grdeficient H-Y TCR-transgenic mice that impairs both MHC class I- and class II-restricted positive selection events.

To examine the consequence of CaMKIV/Gr-deficiency on negative selection, 3.L2tg/CaMKIV/Gr KO mice were crossed with transgenic mice expressing mHEL into which Hb/H9252 (64–76) had been engineered (4). The mHEL/Hb (64–76) transgene is under control of Eα promoter, directing expression into APCs, including those in the thymus. The mHEL/Hb (64–76) protein is well expressed and efficiently processed, enabling presentation of Hb (64–76) in the context of I-Ek. When 3.L2tg mice are crossed with those expressing mHEL/Hb (64–76), 3.L2 TCR+ thymocytes are completely deleted (4). Fig. 5a demonstrates that 3.L2 TCR+ thymocytes of CaMKIV/Gr WT and KO mice were deleted to a similar extent when exposed to mHEL/Hb (64–76), indicating that clonal deletion of 3.L2tg thymocytes by Hb (64–76) was not impaired by CaMKIV/Gr deficiency.

The influence of CaMKIV/Gr deficiency on negative selection was further examined using male H-Y TCR-transgenic mice. In male mice, engagement of the H-Y TCR by the H-Y Ag results in deletion of H-Y TCR+ thymocytes, leading to a drastic reduction in the number of DP and SP thymocytes (31). Examination of thymi of H-Y TCR+ WT and CaMKIV/Gr KO male mice revealed equally reduced cellularity and thymocyte deletion (Fig. 5b). This indicated that CaMKIV/Gr deficiency did not affect negative selection of H-Y TCR+ thymocytes by the H-Y Ag.

Altered selection threshold in CaMKIV/Gr KO mice

Because Hb (64–76) and the H-Y Ag are strong deleting ligands, we sought to examine the effect of CaMKIV/Gr on clonal deletion by weaker ligands. To that end, we employed altered peptide ligands derived from Hb (64–76) in which the asparagine residue at position 72 of Hb (64–76) (N72) has been changed to either isoleucine (I72) or alanine (A72). Both I72 and A72 are antagonists whose relative affinity for the 3.L2 TCR follows the order N72 (WT)/I72/A72 (4). The I72 peptide induces strong negative selection of 3.L2tg thymocytes, whereas the A72 peptide induces weak to moderate negative selection. Both I72 and A72 peptides were engineered into mHEL proteins and expressed in APCs of transgenic animals under control of I-Eα promoter (4). Expression levels of mHEL/I72 and mHEL/A72 were equivalent to those achieved with mHEL/Hb (64–76) (Ref.
Transgenic mice expressing mHEL/I72 (I72tg) and mHEL/A72 (A72tg) were crossed with 3.L2tg/Hb (64–76) × CaMKIV/Gr KO mice. From these plots, the CD4-SP regions (R1) were selected for further analysis of 3.L2 clonotypic TCR expression. The histogram on the left shows overlay of 3.L2tg × mHEL/Hb (64–76) (solid line) and 3.L2tg controls (dotted line). Histogram on the right shows overlay of 3.L2tg × mHEL/Hb (64–76) × CaMKIV/Gr KO (solid line) and 3.L2tg × CaMKIV/Gr KO (dotted line). Results are representative of three pairs of mice. 

To determine whether the increased positive selection in 3.L2tg × A72tg × CaMKIV/Gr KO mice results in the production of functional 3.L2 TCR CD4+ splenocytes, we compared the proliferative responses to Hb (64–76) peptide of splenocytes of 3.L2tg, 3.L2tg × CaMKIV/Gr, 3.L2tg × A72tg, and 3.L2tg × A72tg × CaMKIV/Gr KO. The results, normalized for 3.L2
TCR+CD4+ cell count and shown in Fig. 6d, revealed that splenocytes of 3.L2tg and 3.L2tg x A72tg x CaMKIV/Gr KO mice proliferated equally well in response to Hb64d (64–76) peptide. Importantly, splenocytes of 3.L2tg x A72tg and 3.L2tg x A72tg x CaMKIV/Gr KO mice also proliferated equally well in response to Hb64d (64–76) peptide, indicating that they were functionally equivalent. The presence of the A72tg was associated with a modest shift to the right in the peptide concentration/proliferative response curves of both WT and KO splenocytes expressing either the 3.L2tg or 3.L2tg x A72tg. d. Proliferative responses of splenocytes of the respective strains to Hb64d (64–76). Results were normalized for the number of 3.L2 TCR+ splenocytes present in culture and expressed as cpm/10^6 3.L2 TCR+ splenocytes.

Defective induction of Ca2+-regulated genes in CaMKIV/Gr KO thymocytes

To elucidate mechanisms by which CaMKIV/Gr regulates thymocyte selection, we examined Ca2+-regulated gene expression in thymocytes of CaMKIV/Gr KO mice. Previously, studies using T cell lines have demonstrated that CaMKIV/Gr mediates Ca2+-dependent transcriptional activation of several genes relevant to thymocyte selection. These encode the TNF family members CD40 ligand (CD40L) and TNF-α and the orphan steroid receptor Nur77, all of which have been implicated in negative selection (33–35). Fig. 7a demonstrates that, compared with WT thymocytes, CaMKIV/Gr KO thymocytes expressed markedly lower levels of CD40L, TNF-α, and Nur77 transcripts following stimulation with phorbol ester and Ca2+ ionophore. Transcripts of other Ca2+-regulated genes such as c-fos were modestly decreased, while those of

FIGURE 6. Altered selection threshold in CaMKIV/Gr KO mice. a. Negative selection in 3.L2tg x A72tg mice. Dot plot analysis of CD4 and CD8 staining on thymocytes of 3.L2tg x A72tg and 3.L2tg x A72tg x CaMKIV/Gr KO mice. The histogram on the left shows overlay of 3.L2tg x A72tg (solid line) and 3.L2tg controls (dotted line). Histogram on the right shows overlay of 3.L2tg x A72tg x CaMKIV/Gr KO (solid line) and 3.L2tg x CaMKIV/Gr KO (dotted line). b and c. Frequency of 3.L2 TCR+CD4-SP thymocytes (b) and splenocytes (c) in WT and KO strains expressing either the 3.L2tg or 3.L2tg x A72tg. d. Proliferative responses of splenocytes of the respective strains to Hb64d (64–76). Results were normalized for the number of 3.L2 TCR+ splenocytes present in culture and expressed as cpm/10^6 3.L2 TCR+ splenocytes.

FIGURE 7. Impaired Ca2+-regulated gene expression in CaMKIV/Gr KO thymocytes. a. Northern blot analysis of total RNA isolated from untreated and phorbol ester and calcium ionophore-treated thymocytes of WT, Het, and CaMKIV/Gr KO mice. Results are representative of three separate experiments. b. Normal MAP kinase activation and CREB phosphorylation in CaMKIV/Gr KO thymocytes. Thymocytes of WT and KO mice were stimulated with cross-linked anti-CD3ε mAb and then examined for ERK and CREB phosphorylation by immunoblotting with specific phosphoantibodies. The total amount of ERK and CREB proteins was subsequently determined by immunoblotting with anti-ERK and anti-CREB Abs. Results are representative of three separate experiments.
CaMKIV/Gr promotes thymocyte selection by low-affinity ligands

e-jun were spared. These results revealed a selective impairment of Ca²⁺-regulated gene transcription in CaMKIV/Gr KO thymocytes.

The effects of CaMKIV/Gr on thymocyte selection overlap with those of previously reported lesions in other signaling pathways. In particular, ERK1 and ERK2 have previously been demonstrated to promote positive selection (36–38). To determine whether CaMKIV/Gr deficiency impairs ERK activity, WT and KO thymocytes were stimulated with anti-CD3ε mAb and then examined for ERK1 and ERK2 activation, as evidenced by activation loop-specific phosphorylation of ERK1 and ERK2. Results revealed that ERK activation proceeded normally in CaMKIV/Gr KO thymocytes (Fig. 7b). This indicated that the impact of CaMKIV/Gr deficiency on thymocyte selection was unlikely to be due to impaired ERK activity.

CaMKIV/Gr is also known to be a prominent activator of CREB, which it phosphorylates on the regulatory serine 133 residue (39, 40). Its deficiency is associated with impaired Ca²⁺-dependent CREB phosphorylation in neurons (28, 41). Because CREB has been implicated in regulating thymocyte development (42, 43), we examined whether the effects of CaMKIV/Gr deficiency on thymocyte development correlate with impaired CREB activation. Thymocytes of WT and KO mice were stimulated with anti-CD3ε mAb and then examined for CREB activation, as evidenced by its phosphorylation on serine 133. Results (Fig. 7b) revealed that CREB phosphorylation proceeded normally in CaMKIV/Gr KO thymocytes, indicating that unlike the case of neurons, CREB activation in CaMKIV/Gr KO thymocytes is rescued by other CREB-activating kinase(s).

Discussion

In this work, we provide evidence for an important role for CaMKIV/Gr in thymocyte selection. First, CaMKIV/Gr deficiency was associated with impaired generation of both CD4-SP and CD8-SP thymocytes. The defect involved a block in the transition from DP into SP thymocytes, as evidenced by a corresponding increase in the number of DP thymocytes. This block did not involve lineage commitment per se, but rather trophic signal(s) that promotes maturation of both lineages. Significantly, CaMKIV/Gr KO mice bred onto MHC class I (H-Y)- and class II (3.L2 and Hb9)–restricted TCR-transgenic backgrounds exhibited a similar deficit in the generation of TCR-transgenic SP thymocytes. These results are all consistent with impaired thymocyte-positive selection in CaMKIV/Gr KO mice.

CaMKIV/Gr deficiency did not affect negative selection by high-affinity peptide ligands. This was evidenced by the effective deletion of CaMKIV/Gr KO thymocytes bearing either MHC class I (H-Y)- or II (3.L2)-restricted transgenic TCR upon exposure to their specific Ags. However, by using altered peptide ligands that vary in their affinity for the 3.2 L TCR, it could be demonstrated that CaMKIV/Gr deficiency altered the outcome of negative selection by a low-affinity ligand. This was evidenced by the abrogation in 3.2tg x CaMKIV/Gr KO mice of weak negative selection by the A72 peptide, which instead acted to promote positive selection. Because positive selection is normally an attribute of peptides of lower affinity than A72 for the 3.2 L TCR, this indicated that CaMKIV/Gr deficiency rendered the A72 peptide functionally equivalent to a peptide of lower affinity for the 3.2 L TCR.

The quantitative/avidity model of thymocyte selection predicts that a majority of peptides encountered during development have either no affinity (null) or low affinity (positively selecting) for a given TCR, while a small population of peptides exhibits high enough affinity to initiate negative selection (3, 44). Given that CaMKIV/Gr KO mice suffer impairment of selection processes typically driven by low-affinity peptide/TCR interactions including positive selection and weak negative selection, it can be concluded that CaMKIV/Gr-regulated pathways function to sensitize thymocytes to selection by lower-affinity peptides. This may be due to a requisite role for CaMKIV/Gr in a subset of signaling events triggered by low-affinity peptides. Accordingly, CaMKIV/Gr deficiency raises the threshold for selection such that a large number of low-affinity peptides that normally induce positive selection are rendered functionally null, leading to impaired positive selection. Of the smaller group of peptides that mediate negative selection, the effectiveness of those at the lower end of the affinity spectrum is attenuated, leading to impaired weak negative selection. Peptides at the threshold of negative selection such as A72 may instead promote positive selection in the context of CaMKIV/Gr deficiency. However, this is insufficient to rescue the defect in positive selection possibly due to the low frequency of such peptides. Only negative selection by a minority of peptides at the high end of the affinity spectrum, such as the H-Y Ag and Hb9 (64–76), is spared.

The expression profile and activation mechanism of CaMKIV/Gr make it particularly suited for promotion of thymocyte selection by low-affinity peptides. CaMKIV/Gr levels are highest in DP thymocytes, which are the target of thymocyte selection events. CaMKIV/Gr is itself a component of an independent CaMK cascade that includes the upstream activators CaMKKα and CaMKKβ (45). Signal amplification by this cascade may allow CaMKIV/Gr to be activated at lower Ca²⁺ concentrations, such as those triggered by low-affinity ligands. CaMKIV/Gr deficiency did not impair transient, high-intensity activation of other kinase cascades involved in thymocyte selection, such as those of the mitogen-activated protein (MAP) kinases. A more subtle crosstalk between the CaMK and MAP kinase cascade cannot however be ruled out and requires further investigation.

The thymic phenotype observed in CaMKIV/Gr KO mice contrasts with that previously reported for mice expressing a dominant-negative (DN) CaMKIV/Gr mutant in thymocytes (46). Thymi of the latter mice were profoundly hypocellular, and their T cells proliferated poorly upon stimulation with anti-CD3 mAbs. The discrepant phenotype between the CaMKIV/Gr KO and DN CaMKIV/Gr KO mouse may be due to inhibitory interference by the DN CaMKIV/Gr mutant with other protein kinases that ameliorate the impact of CaMKIV/Gr deficiency on thymic development. These are most likely to be other components of the CaMK cascade itself. Both CaMKKα and CaMKKβ physically interact with CaMKIV/Gr by means of a specialized domain (47). Hence, overexpression of the DN CaMKIV/Gr mutant is likely to tie up both upstream activating kinases in complexes with the inactive mutant, leading to inhibition of their CaMKIV/Gr-dependent and independent functions. The global role of the CaMK cascade in thymic selection is currently being addressed using mutant mice lacking different component kinases.

A principal function of CaMKIV/Gr is Ca²⁺-dependent transcriptional activation (45). Consistent with this function is the decreased transcription in CaMKIV/Gr KO thymocytes of several Ca²⁺-regulated genes. Some affected genes such as CD40L, TNF, and Nur77 have been previously implicated in negative selection (33–35), suggesting that their defective transcription may contribute to the impaired weak negative selection in CaMKIV/Gr KO mice. Altered transcription of other currently unknown genes may similarly contribute to impaired positive selection in CaMKIV/Gr KO mice.

CaMKIV/Gr regulates several transcriptional activators, coactivators, and corepressors, including CREB (39, 40), p300/CREB-binding protein (48), myocyte enhancer factor 2 (MEF2) (27), and histone deacetylases (49). CaMKIV/Gr deficiency appeared to selectively impact some factors but not others. TCR-triggered CREB...
phosphorylation proceeded normally in CaMKIV/Gr-deficient thymocytes, suggesting that this pathway was rescued by the action of other CREB kinases. In contrast, Ca2+ activation of MEF2 factors appeared impaired, as evidenced by decreased transcription of Nur77, a prototypic, MEF2-regulated immediate-early activation gene (27, 50). Because evaluation of Ca2+-dependent gene transcription was performed under conditions approximating strong TCR/CD3 signaling induced by high-affinity ligands, a more dramatic impact of CaMKIV/Gr deficiency on select Ca2+-regulated transcriptional events may be revealed by using low-affinity ligands or surrogate stimulation paradigms. Such studies, currently ongoing, may also be informative into the role of individual Ca2+-regulated factors in mediating the effects of CaMKIV/Gr on thymocyte selection.

Finally, the interplay between the CaMK cascade and other Ca2+ signaling pathways in directing the selection outcome in thymocytes merits attention. Some pathways, such as the CaMK cascade (this study) and the calcineurin/NF-AT module (16–20), appear to be preferentially employed by thymocytes to decode Ca2+ signals associated with positive and weak negative selection. Others, such as the NH2-terminal Jun kinase, may be recruited by stronger Ca2+ responses associated with negatively selecting ligands (51). It will be important to elucidate the different affinity thresholds at which these pathways are recruited and how different pathways interact to achieve a particular selection outcome.

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References


