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Calcium/Calmodulin-Dependent Protein Kinase IV Suppresses IL-2 Production and Regulatory T Cell Activity in Lupus

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The activity of calcium/calmodulin-dependent protein kinase IV (CaMK4) is increased in T cells from patients with systemic lupus erythematosus (SLE) and has been shown to reduce IL-2 production by promoting the effect of the transcriptional repressor cAMP responsive element modulator-α on the IL2 promoter. In this article, we demonstrate that T cells from MRL/lpr mice display increased levels of CaMK4 in the nucleus, and that genetic deletion of Camk4 results in improved survival. We demonstrate that absence of CaMK4 restores IL-2 production, curbs increased T cell activation, and augments the number and activity of regulatory T cells. Analogously, silencing of CaMK4 in T cells from patients with SLE increases the expression of FoxP3 on stimulation in the presence of TGF-β. Our results demonstrate the importance of the serine/threonine kinase CaMK4 in the generation and function of regulatory T cells in patients with SLE and lupus-prone mice, and its potential to serve as a therapeutic target.

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CD11c (HL3; BD Pharmingen), CD19 (1D3; eBioscience), CD25 (PC61; eBioscience), CD44 (DM7; BD Pharmingen), CD62L (MEL14; BD Pharmingen), or F4/80 (BM8; eBioscience) for 30 min at 4°C. For intracellular staining of FoxP3 (MF23; BD Biosciences) and IL-2 (JES6-5H4; BioLegend), the FoxP3-Staining Buffer Set (fixation/permeabilization and permeabilization buffers; eBioscience) and BD cytofix/cytoperm plus (with Golgi Plug) intracellular staining kits (BD Biosciences) were used according to the manufacturer’s protocol. Total cell numbers were determined by counting live cells. Absolute cell numbers were calculated on the basis of the percentage of each population and represented as median.

**Cell sorting**

Cell sorting was performed in a FACS Aria II cell sorter (BD Biosciences). Splenocytes from MRL/lpr mice were isolated, and a minimum of 1 x 10⁶ total splenocytes was incubated with Abs against CD3, CD4, CD8, CD19, CD11c, and F4/80 for 30 min at 4°C. Cells were sorted into six different populations, CD3⁺CD4⁻CD8⁻, CD3⁺CD4⁺CD8⁻, CD3⁻CD4⁺CD8⁺, CD3⁻CD19⁻, CD3⁻CD11c⁻, and CD3⁻F4/80⁺. The purity of the sorted cells populations ranged from 94 to >99%.

**RNA isolation and real-time RT-PCR**

Total mRNA was isolated from spleen cells using the RNeasy Mini Kit (Qiagen). cDNA was produced using random primers from an equal amount of RNA. Real-time RT-PCR for mouse Il2, mouse Camk4, mouse Gapdh, human FOXP3, and human Actb were performed (Light Cycler 480; Roche, Indianapolis, IN) with ABI TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA). Gene expression was assessed by comparative cycle threshold method.

**Polyclonal activation and suppression assay**

CD3⁺CD4⁺CD25⁻ T cells (responder T cells) and CD3⁺CD4⁺CD25⁺ T (Treg) cells were isolated from spleens by FACS sorting. CD4⁺CD25⁻ T cells were labeled with 0.5 μM CFSE (Invitrogen/Molecular Probes) for 10 min at 37°C. CFSE-labeled CD4⁺CD25⁺ T cells were cultured in 96-well round-bottom plates at 2 x 10⁶ cells/well in the presence of varying amounts of CD4⁺CD25⁺ cells and stimulated with plate-bound goat anti-hamster Abs (MP Biomedicals), soluble anti-CD3 (0.25 μg/ml, clone 145-2C11; BioLegend) and anti-CD28 (0.5 μg/ml, clone 37.51; BioLegend). After 5 d, cells were harvested and stained for surface markers, and CFSE signal of gated lymphocytes was analyzed by flow cytometry.

**Isolation of cells and cell culture**

Naïve CD4 T cells were isolated by magnetic cell sorting (CD4⁺CD62L⁺ T cell isolation kit II; Miltenyi Biotec). Possorting purity was >95%. Cells were stimulated in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 50 μM 2-ME, 50 μM penicillin G, and 50 μg/ml streptomycin sulfate with plate-bound goat anti-hamster Abs, soluble anti-CD3 (0.25 μg/ml, clone 145-2C11), and anti-CD28 (0.5 μg/ml, clone 37.51). In vitro Treg differentiation assays were performed by stimulating cells with plate-bound anti-hamster Abs (MP Biomedicals), soluble anti-CD3 (0.25 μg/ml, clone 145-2C11, BioLegend) and anti-CD28 (0.5 μg/ml, clone 37.51; BioLegend). After 5 d, cells were harvested and stained for surface markers, and CFSE signal of gated lymphocytes was analyzed by flow cytometry.

**ELISA**

Splenical naïve CD4⁺CD62L⁺ T cells (5 x 10⁶ cells/sample) were stimulated as described earlier. After 24 h, IL-2 was measured in supernatants by ELISA (R&D Systems). Serum anti-dsDNA Ab concentration was detected by mouse anti-dsDNA IgG ELISA kit (Alpha Diagnostic).

**Western blotting**

Splenocytes of MRL/lpr or MRL/lpr+Ccamk4⁻/⁻ mice were lysed in RIPA buffer at 4°C for 30 min. After centrifugation (14,000 rpm; 30 min; 4°C), supernatants were collected and nuclei were isolated using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). An identical amount of protein from each lysate (5 μg/well) was separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen). Proteins were transferred to a nitrocellulose or polyvinylidene difluoride membrane, which was subsequently blocked for 1 h using 2% BSA in PBS and incubated at room temperature with anti-Camk4 (Cell Signaling), anti-CRE (IMAGEX) anti–phospho-Ser274 CREM, anti–phospho-Ser274 CREM (Novus), and anti-actin (Sigma). The membranes were washed with TBS-T and incubated with a 1:5000 dilution of goat anti-rabbit IgG or donkey anti-goat IgG coupled with HRP (Jackson ImmunoResearch, West Grove, PA). The ECL system (Amersham, Buckinghamshire, U.K.) was used for detection.

**Human SLE T cells**

T cells were obtained from the peripheral blood of patients with SLE, as described previously under an Institutional Review Board-approved protocol and transfected with CAMK4-specific or control siRNA as described previously (8). After 24 h, cells were stimulated with plate-bound goat anti-mouse Abs (Chemicon Millipore), soluble anti-CD3 (5 μg/ml, clone OKT3; BioXcell), anti-CD28 (2.5 μg/ml, clone 28.2; BioLegend), and TGF-β1 (5 ng/ml; R&D Systems), and cultured for 96 h.

**Statistical analyses**

Student two-tailed t tests and Mann–Whitney U tests were used. Mouse survival was analyzed by the Kaplan–Meier method and the log-rank test. Statistical analyses were performed in GraphPad Prism 5.01 software. A p value <0.05 was considered significant.

**FIGURE 1.** Camk4 activity is increased in T cells from MRL/lpr mice. (A) Camk4 expression was quantified using real-time RT-PCR in RNA extracts from T cell subsets (CD4⁺, CD8⁺, CD4⁺CD8⁻), B cells (CD19⁺), dendritic cells (CD11c⁺), and macrophages (F4/80⁺) isolated by FACS sorting from 16-wk-old MRL/lpr mice. (B) Camk4 expression was quantified by real-time RT-PCR in spleen cells from MRL/lpr control mice and from 6- and 16-wk-old MRL/lpr mice. *p < 0.05. (C) Camk4 was detected by Western blot in the nuclear and cytoplasmic fractions of spleen cell lysates from MRL/lpr, MRL/lpr, and MRL/lpr+Ccamk4⁻/⁻ mice. Data are representative of two independent experiments, each using four mice per group.

**FIGURE 2.** Camk4 deficiency prolongs survival of MRL/lpr mice. Survival of MRL/lpr and MRL/lpr+Ccamk4⁻/⁻ are depicted. Mice were observed until 32 wk of age (n = 10 mice/group).
Results

CaMK4 is found in the nuclei of MRL/lpr cells

Activity of the serine/threonine kinase CaMK4 is abnormally increased in T cells from patients with SLE (8). Activation of CaMK4 induces its migration into the cell nucleus (7). Accordingly, higher levels of CaMK4 are found in the nuclei of T cells from patients with SLE than in healthy control subjects. Increased CaMK4 activity has been linked to a number of phenotypic abnormalities in SLE T cells, including increased activation of the transcriptional factor CREM-α (8) and decreased IL-2 production (8). Because IL-2 production by CD4 T cells is similarly decreased in MRL/lpr mice (11) that develop a lupus-like disease, we decided to investigate whether the expression and cellular localization of CaMK4 was abnormal in these mice, to determine whether they represent a useful system to study the consequences of increased CaMK4 activity in systemic autoimmunity.

As shown in Fig. 1A, high levels of Camk4-encoding transcripts were found in T lymphocytes, particularly CD4+ T cells, of MRL/lpr mice. In contrast, levels of Camk4 mRNA were scarce in spleen-derived B cells, dendritic cells, and macrophages. We compared the mRNA levels of Camk4 in spleen cells from 8- (before disease onset) and 16-wk-old (established disease) MRL/lpr mice. Camk4 expression in 8-wk-old MRL/lpr mice did not differ from that of the control strain MRL/MPJ. In contrast, 16-wk-old MRL/lpr mice had significantly higher levels of Camk4 measured by quantitative PCR (Fig. 1B). Because activated CaMK4 migrates to the cell nucleus, we investigated its abundance in the cytosolic and nuclear fractions of spleen cells isolated from MRL/lpr, MRL/MPJ, and MRL/lpr.Camk4-/- mice. High levels of CaMK4 were detected in the nuclei of MRL/lpr spleen cells. This contrasted sharply with the cellular distribution of CaMK4 in spleen cells of the Fas-intact control strain MRL/MPJ, where virtually all CaMK4 was found in the cytoplasmic fraction of the cell lysate (Fig. 1C).

These results indicate that CaMK4 expression in the spleen is mainly restricted to T cells and demonstrate that, as in patients with SLE (8), CaMK4 levels and activity are increased in diseased MRL/lpr mice.

FIGURE 3. CaMK4 deficiency limits lymphoid cell activation and proliferation. (A) Representative photograph of the spleens and axillary lymph nodes from 16-wk-old MRL/MPJ, MRL/lpr, and MRL/lpr.Camk4-/- mice. (B) Absolute numbers of spleen and axillary lymph node cells are shown. (C) Absolute numbers of splenic CD4+, CD8+, and double-negative (CD4-CD8-) T cells. (D) Abundance of naive (CD44lowCD62Lhigh) and activated (CD44highCD62Llow) CD4 T cells (shown as percentage of CD4+ T cells). All experiments were performed in 16-wk-old female mice. Each dot represents an individual mouse. Horizontal bars indicate mean for each group. ▲p < 0.05, ▲▲p < 0.01.
CaMK4 deficiency ameliorates disease and reduces mortality in MRL/lpr mice

Pharmacological inhibition or genetic deletion of CaMK4 reduces autoantibody production and reduces progression of kidney disease in MRL/lpr mice (9, 10). We confirmed those results and found that the effect of Camk4 deficiency reduces the mortality of MRL/lpr mice in a statistically significant manner. The survival rate of MRL/lpr.Camk4−/− mice was 89% at 32 wk of age compared with only 25% in the MRL/lpr group (p < 0.05; Fig. 2). As previously shown, the severity of the glomerulonephritis, as well as the levels of C3 deposits in the kidney and the extent of skin injury were notably decreased in MRL/lpr.Camk4−/− mice (Supplemental Fig. 1).

T and B cell activation and proliferation are prominently increased in MRL/lpr mice (12) and occur simultaneously to the development of autoantibodies and organ damage. In a previous work, we demonstrated that CaMK4 facilitates the proliferation of mesangial cells in the renal glomeruli of MRL/lpr mice (10). To determine whether CaMK4 is also involved in the augmented lymphoid activation and proliferation of MRL/lpr mice, we analyzed the phenotype of different T and B cell subsets using multicolor flow cytometry. CaMK4 deficiency was associated with a significant decrease in spleen size and cellularity (p = 0.01) in 16-wk-old mice. In contrast, absence of CaMK4 did not affect in a statistically significant manner the size and total cell content of the axillary lymph nodes (Fig. 3A, 3B). Importantly, the abundance of CD4+CD8− (double-negative) T cells and activated CD4+ (CD44+CD62Lhi) T cells was significantly reduced in the absence of CaMK4 (Fig. 3C, 3D). A reciprocal increase in naive CD4+ (CD44−CD62Lo) T cells was likewise observed (Fig. 3D).

These results suggest that CaMK4 is involved in the activation and proliferation of lymphoid cells in the MRL/lpr mice, because its deficiency decreases the numbers of activated cells.

CaMK4 deficiency restores IL-2 production by MRL/lpr CD4 T cells

Transcription of Il2 upon T cell stimulation is abnormally decreased in patients with SLE (13, 14) and in MRL/lpr mice (15, 16). Although several molecular alterations have been linked to this phenotype (17), an imbalance between the repressor CREM and the enhancer CREB contributes to decreased Il2 transcription (4). Increased binding of CREM to the Il2 promoter has been shown to impair IL-2 production in T cells from patients with SLE in an analogous manner to its effects in anergic cells (4, 8). Because CaMK4 modulates the activity of CREM (4, 18), we hypothesized that Camk4 deficiency would affect the production of IL-2 by naive CD4 T cells. As shown in Fig. 4, absence of CaMK4 completely restored the production of IL-2 in MRL/lpr mice. This was evident at the mRNA and protein levels (ELISA and intracellular flow cytometry).

To determine whether the effect on IL-2 production was exerted through CREM activation, we analyzed the phosphorylation of CREM in MRL/lpr and MRL/lpr.Camk4−/− mice after T cell activation. As shown in Fig. 4D, phospho-CREM was detected in MRL/lpr cells after 20 min of interaction with anti-CD3 and anti-CD28. In sharp contrast, no phospho-CREM could be detected in Camk4 null cells.

CaMK4 controls Treg differentiation and function

IL-2 is intimately linked to the maintenance and function of Tregs (19). In fact, mice deficient in IL-2 or in critical components of its receptor experience development of a lethal autoimmune disease caused by a lack of Tregs (20). Numerical and functional Treg deficiencies have also been described in patients with SLE (21), and Tregs from MRL/lpr mice have been reported to be functionally abnormal (22). We reasoned that the decrease in spontaneous T cell activation observed in the Camk4-deficient MRL/lpr mice could be associated with restored Treg function, perhaps facilitated by the rescue of the IL-2 production capacity.

To address this, we quantified the abundance of Tregs in the spleens of MRL/lpr and MRL/lpr.Camk4−/− mice. Tregs (CD4+CD25+Foxp3+) were significantly increased in Camk4−/− mice when compared with Camk4-sufficient mice (10.3 ± 0.6 versus 7.2 ± 0.9%; p < 0.05; Fig. 5A). Moreover, expression of Foxp3 at the protein level was increased in Tregs from MRL/lpr.Camk4−/− mice compared with Tregs from MRL/lpr mice (Fig. 5B). To determine whether CaMK4 modulates the differentiation of Tregs from naive CD4 T cells, we quantified induction of Tregs under
CaMK4 regulates the generation and function of Tregs. To this end, CD4+CD25+ T cells isolated by FACS sorting were stimulated with anti-CD3 and anti-CD28 in the presence of TGF-β, anti–IFN-γ, and anti–IL-4 for 5 d. Cells activated in the absence of TGF-β served as controls. As shown in Fig. 5E, Treg induction was significantly increased in cells isolated from MRL/lpr.Camk4−/− mice (Camk4+/+ 13.7% versus Camk4−/− 19.9%; p < 0.05). This effect was probably mediated through IL-2 because the addition of exogenous IL-2 to the culture was able to increase the yield of CD25+FoxP3+ T cells generated from Camk4−-sufficient mice to the same level observed in cells from Camk4−-deficient animals (Fig. 5E).

The suppressive capacity of Camk4−-deficient and -sufficient Tregs was evaluated in in vitro coculture assays. As shown in Fig. 5C, Tregs from MRL/lpr.Camk4−/− mice suppressed more efficiently the proliferation of CD4+CD25− T cells from Camk4−-sufficient T cells than Tregs from wild type mice.

Taken together, our results suggest that by rescuing IL-2 production, CaMK4 deficiency restores Treg numbers and function that curb spontaneous lymphoid activation.

Silencing of CaMK4 results in overexpression of Tregs in patients with SLE

To determine the relevance of our findings to human SLE, we analyzed the effect of CaMK4 inhibition in T cells from patients with SLE. By using RNA interference, we successfully knocked down CaMK4 expression in T cells (Fig. 6A, 6B). To determine the effect of CaMK4 inhibition in FoxP3 expression, we stimulated cells transfected with Camk4-specific or with control siRNA with anti-CD3, anti-CD28, and TGF-β. As shown in Fig. 6, CaMK4 inhibition caused a significant increase in the expression of Foxp3 gene as determined by real-time RT-PCR (Fig. 6C). Accordingly, CaMK4 inhibition significantly augmented the percentage of CD25+FoxP3+ T cells (Fig. 6D). These results indicate that, as in MRL/lpr mice, CaMK4 is a negative regulator of Tregs in patients with SLE.

Discussion

Decreased IL-2 production upon T cell activation represents a significant cytokine aberration in SLE (13, 14) that has been recognized in MRL/lpr mice (15, 16). Our work identifies CaMK4 as a key molecule involved in the downregulation of IL-2 in MRL/lpr mice and proves that rescue of IL-2 production has beneficial immunological and clinical consequences. In summary, CaMK4 promotes CREM-α–mediated suppression of IL-2 production and Treg function associated with increased presence of activated T cells and lupus-related pathology.

Complete absence of IL-2 or key elements of its receptor and associated signaling molecules cause lethal autoimmune disease...
CaMK4 regulates FoxP3 in T cells from SLE patients. (A) T cells from four patients with SLE were transfected with either control siRNA or Camk4-specific siRNA. A representative image of CaMK4 expression (Western blot) at 24 and 48 h after transfection is shown. (B) Cumulative data from four experiments. (C and D) Twenty-four hours after transfection, cells were either left unstimulated (white bars) or stimulated with anti-CD3 and anti-CD28 plus TGF-β (5 ng/ml; black bars). After 96 h, FoxP3 expression was measured by real-time RT-PCR (C) or flow cytometry (D). *p < 0.05, **p < 0.01.

This syndrome is caused by lack of Tregs that depend on IL-2 signaling for their maintenance (20). Reduced Treg numbers (21, 25) and function (26) have been reported in patients with SLE, and these defects have been associated with increased lupus disease activity (21). Although reduced IL-2 secretion has been proposed to underlie the SLE Treg defect (27), other causes, in particular, chronic inflammation, are also thought to contribute (28). Moreover, because the magnitude of Treg deficiency is variable and not absolute, its contribution to SLE has been debated. Administration of IL-2 through an IL-2–encoding vaccinia virus was able to decrease disease severity in MRL/lpr mice (29). However, the mechanism underlying disease amelioration was not sought in this early report (29). Two recent clinical trials performed in patients with immune-mediated diseases (hepatitis C virus-induced vasculitis and chronic graft-versus-host disease) demonstrated that administration of exogenous IL-2 can indeed have an immune-modulating effect associated with an increase in the numbers of Tregs (30, 31). By studying the effects of the deficiency of Camk4 in mice with a chronic inflammatory autoimmune disease, we have produced data that indicate that CaMK4 is a central link between inflammation and decreased IL-2 production, and that by eliminating this single kinase, IL-2 production is completely restored. Importantly, restoration of IL-2 has vast consequences in the immune system. It restricts the spontaneous activation of T cells reducing the fraction of CD4+CD8−T cells, and it increases the abundance of FoxP3+ Tregs. The relevance of these changes is reflected in a significant increase in survival. Thus, our results support the concept that reduced IL-2 secretion is directly associated with SLE pathogenesis, and that its restoration could be beneficial through effects in Treg number, Treg function, and decreased T cell activation.

The fact that young MRL/lpr mice have normal levels of Camk4 suggests that this defect is not associated with the lack of Fas or with the genetic background that causes susceptibility to autoimmunity in this particular strain. Rather, increased CaMK4 levels and activation in diseased MRL/lpr mice may result from the autoimmune and/or inflammatory changes that occur in these mice when disease develops. We have previously reported that autoantibodies in sera from patients with SLE can induce CaMK4 activation and alter IL-2 production (8). Thus, CaMK4 activation in MRL/lpr mice could be caused in a similar fashion. The fact that CaMK4 deficiency abrogates lupus-associated pathology indicates that, although high CaMK4 activity is not a primary defect, it certainly contributes to disease development. Our data indicate that absence of CaMK4 increases the numbers and function of Tregs. This effect is probably secondary to the restoration of IL-2 production because we were able to mimic it by adding exogenous IL-2 in the in vitro Treg differentiation assays. However, we cannot rule out whether CaMK4 exerts a direct suppressive effect on FoxP3 expression that could account for higher FoxP3 levels in Tregs of Camk4-deficient mice. We should point out that for the functional assays, we used Tregs sorted by the expression of CD25, a population that may also include effector CD4+ T cells.

We have previously reported biochemical evidence that shows that CaMK4 can directly inhibit IL-2 production by activating the repressor activity of CREM-α (8). The results presented in this article suggest that at the in vivo level, Camk4 deficiency may also rescue IL-2 production by decreasing the number of activated T cells and increasing the pool of naïve T cells that are more efficient IL-2 producers (32).

In this work, as well as in previous reports from our group (8, 10), we have identified CaMK4 as a kinase that becomes activated in the setting of chronic inflammation. CaMK4 activity was linked to high mesangial cell proliferation rates in MRL/lpr mice, and its ablation protected the kidney from glomerulonephritis (10). In this article, we have shown that CaMK4 also facilitates T cell activation and proliferation in MRL/lpr mice, and in its absence T cell activation is reduced. Human T cells incubated with sera from patients with SLE show enhanced CaMK4 expression (8). The identification of the elements that regulate CaMK4 activation and that explain its link to inflammation will be sought in future work.

In conclusion, we have provided evidence that CaMK4 plays a central role in the development of autoimmunity in the MRL/lpr mouse. Its increased activation promotes T cell activation and organ damage. These effects are mediated by the suppression of IL-2 production and by reducing the numbers and function of Tregs. Our data indicate that IL-2-deficient production may represent a key molecular defect in the setting of SLE amenable of being corrected by the inhibition of CaMK4.

Disclosures
The authors have no financial conflicts of interest.

References


