Cutting Edge: Calcium/Calmodulin-Dependent Protein Kinase Type IV Is Essential for Mesangial Cell Proliferation and Lupus Nephritis

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Cutting Edge: Calcium/Calmodulin-Dependent Protein Kinase Type IV Is Essential for Mesangial Cell Proliferation and Lupus Nephritis

Kunihiro Ichinose,* Thomas Rauen,* † Yang-Taung Juang,* Katalin Kis-Toth,* Masayuki Mizui,* Tomohiro Koga,* and George C. Tsokos*

Renal involvement in systemic lupus erythematosus remains a major cause of morbidity and mortality. Although immune parameters that instigate renal damage have been characterized, their link to local processes, which execute tissue damage, is poorly understood. Using genetic-deletion and pharmacological-inhibition approaches, we demonstrated that calcium/calmodulin-dependent protein kinase type IV, which contributes to altered cytokine production in systemic lupus erythematosus patients, controls spontaneous and platelet-derived growth factor-stimulated mesangial cell proliferation and promotes IL-6 production through AP-1. Our studies identified calcium/calmodulin-dependent protein kinase type IV as a valuable treatment target for lupus nephritis and point out the importance of local kidney factors in the expression of tissue damage that, if properly targeted, should enhance clinical benefit and limit toxicity. The Journal of Immunology, 2011, 187: 000–000.

Lupus nephritis is still the major cause of morbidity and mortality in patients with systemic lupus erythematosus (SLE) (1). IL-6 can be produced by mesangial cells (MCs) and was reported to orchestrate the cytokine network of glomerular inflammation. There is clear evidence that IL-6 is involved in mesangial proliferation and pathogenesis of lupus nephritis (2–5). The expression of various transcription factors, such as AP-1, CREB, and NF for IL-6, was reported to control IL6 promoter activity (6–8). Among them, AP-1 has been implicated in transcriptional regulation of a wide range of genes participating in cell survival, proliferation, and apoptosis (9–11). The multifunctional calcium/calmodulin-dependent protein kinase type IV (CaMKIV) belongs to a family of serine/threonine protein kinases that regulates autoimmunity and cell proliferation (12–14). A small molecule inhibitor of CaMKIV, KN-93 mitigates disease development in lupus-prone mice by suppressing cytokine production and costimulatory molecule expression in lymphocytes (15). In this report, we provide evidence that pharmacologic inhibition or genetic depletion of CaMKIV in lupus-prone MRL/lpr mice results in decreased mesangial IL-6 production, reduced MC proliferation, and less kidney damage. Our data suggest a prominent role for CaMKIV in the expression of systemic autoimmunity, as well as that of local renal damage.

Materials and Methods

Mice

Female MRL/lpr, Camkiv<sup>Camk4<sup>tm1Tch</sup></sup>J, and MRL/MpJ mice were purchased from Jackson Laboratory. MRL/lpr, Camkiv<sup>Camk4<sup>tm1Tch</sup></sup>J mice were generated on an MRL/lpr background. Experiments were approved by the Institutional Animal Care Committee of Beth Israel Deaconess Medical Center. Measurement of anti-dsDNA Ab levels were performed, as described previously (15). Proteinuria was measured in a semiquantitative manner, as described before (15). Briefly, mice in each group (n = 4) were placed together overnight in a Nalgene metabolic cage to collect urine. This procedure was repeated in two independent experiments, so that the presented data display the average from a total of eight mice/group. Kidneys from 16-wk-old mice were fixed in formalin, paraffin sections were stained with periodic acid-Schiff, and renal lesions were evaluated according to previously described criteria (16, 17). Scoring was performed blindly by a nephropathologist.

Primary culture of MCs

Primary MCs were isolated according to Allam et al. (18), and purity of isolated MCs was assessed by morphologic characteristics, positivity for smooth muscle actin (>99%), and negativity for cytokeratin 18 (>99%). Cultured MCs were used for experiments between passages three and seven. MCs were plated in 12- or 6-well plates and serum starved for 24 h before experiments were performed. Cells were treated with 20 ng/ml platelet-derived growth factor (PDGF)-BB (PeproTech) for 24 h. As indicated, cells were treated with KN-93 (20 μM) for 48 h prior to addition of PDGF-BB. For RNA and protein analyses, EMSAs, and luciferase experiments, MCs were pooled from five mice/group, and each experiment was performed in two or three independent replicates.

Immunoblotting

Briefly, MCs were homogenized in radio immunoprecipitation assay buffer at 4°C for 30 min. After centrifugation (14,000 rpm; 30 min; 4°C), supernatants were collected. The following polyclonal rabbit Abs were used for

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The online version of this article contains supplemental material.

Abbreviations used in this article: CaMKIV, calcium/calmodulin-dependent protein kinase type IV; MC, mesangial cell; PDGF, platelet-derived growth factor; SLE, systemic lupus erythematosus.
immunoblotting: anti-CDK2, anti-cyclin D1, anti-CaMKIV (all from Cell Signaling), anti–c-Jun (Santa Cruz), anti–histone-H3 (Abcam), and anti-actin (Sigma).

**RNA extraction and PCR**

Primary MCs were homogenized, total RNA was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was generated using the Reverse Transcription kit (Promega). PCR primers were as follows: IL-6: 5’-CCGGAGAGGA-GACTTCACAG-3’ (forward) and 5’-CCAGTTTGGTAGCATCCATC-3’ (reverse) and CaMKIV: 5’-TCACATGGACACTGCTCAGA-3’ (forward) and 5’-TGCATCTTTCTCCACCTCCT-3’ (reverse). 18S rRNA primers were reported previously (15).

**IL-6 ELISA**

A total of 400,000 primary MCs was plated on six-well plates and serum starved for 24 h. Then, cells were pretreated with KN-93 (20 μM) for 48 h before the addition of PDGF-BB (20 ng/ml; 24 h). IL-6 concentrations were detected with a commercial ELISA kit (R&D Systems).

**Cell cycle analyses**

MCs were trypsinized, washed twice with PBS, fixed in cold 95% ethanol, and stored at 4˚C until use. Before flow cytometric analysis, cell pellets were washed and resuspended in a solution of RNase (0.5 mg/ml) in PBS and incubated at 37˚C for 20 min. Then, propidium iodide (40 μg/ml) was added for 30 min. Stained cells were analyzed on FACScan (BD Biosciences).

**FIGURE 1.** CaMKIV deficiency improves lupus pathology. Mean scores for glomerular injury (A), tubular damage (B), and perivascular lymphocyte infiltration (C) from MRL/MpJ, MRL/pr, and MRL/pr.Camkiv2/2 mice. D, Proteinuria was quantified weekly starting from 8 wk of age. Anti-dsDNA IgG Abs from 8-wk-old (E) and 16-wk-old (F) mice of each group were detected by ELISA (n = 4–8 mice/group).

**FIGURE 2.** CaMKIV suppresses cell cycle arrest of lupus MCs. A and B, MCs were stained by propidium iodide, and cell cycle analysis was performed by flow cytometry. A total of 400,000 MCs derived from MRL/MpJ and MRL/pr mice was treated or not with KN-93 for 48 h and stimulated with or without PDGF-BB for 24 h. Cumulative flow cytometry data (percentage of cells in each cell cycle phase) are shown for unstimulated (A) and PDGF-BB–stimulated (B) MCs. Values are the mean ± SD (n = 4–5 mice/group) of three independent replicates. C, A total of 400,000 MCs derived from MRL/MpJ, MRL/pr, and MRL/pr.Camkiv2/2 mice was stimulated or not with PDGF-BB for 24 h. Cumulative flow cytometry data (percentage of cells in each cell cycle phase) are shown. Values represent mean ± SD (n = 4–5 mice/group) performed in three independent replicates.
Data were acquired using CellQuest software (BD Biosciences); ≥10,000 events were collected for each graph. Data analysis was performed with FlowJo version 7.6.1 (Tree Star).

**Luciferase assays**

Mouse IL6 promoter luciferase plasmid (in pGL3-Basic vector; Invitrogen) was kindly provided by Dr. David L. Allen (University of Colorado, Denver, CO). Transient transfections were performed in primary MCs (seeded at 1 × 10⁵ cells/well) by using 1 μg reporter DNA, 10 ng pRTK plasmid/transfection, and 2 μl Lipofectamine 2000 (InvivoGen). Twenty-four hours after transfection, cells were incubated or not with PDGF-BB (20 ng/ml) for another 24 h. Luciferase activities in the cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega). Experiments were repeated at least four times. Values in the bar graphs are given as mean ± S.D.

**EMSA**

A total of 500,000 MCs was used for preparation of nuclear protein extracts, as described before (21). A dsDNA probe harboring the AP-1 site (−327) of the murine IL6 promoter (5'-AGTGCAGTCATTTTAAAG-3') was radiolabeled with γ-32P-ATP using a T4-polynucleotide kinase. EMSA was performed, as described before, using 5 μg nuclear protein and 1 μg poly(deoxyguanylic-deoxycytidylic) acid sodium salt per reaction (19). Unlabeled AP-1 probe was used for competition assays in 50- and 100-fold molar excess, as indicated.

**Densitometries and statistical analyses**

Densitometries of Western blot and PCR images were performed using Image J software (National Institutes of Health). The Kruskal–Wallis test was used to determine statistical significance (*p ≤ 0.05, **p ≤ 0.01).

**Results and Discussion**

In our efforts to prove the importance of CaMKIV in the pathogenesis of SLE and lupus nephritis, we transferred the Camkiv null locus into the lupus-prone mouse strain MRL/lpr (20) (Supplemental Fig. 1A, 1B). Total numbers of splenocytes, peripheral lymph node cells, and major B and T cell compartments were not different between MRL/lpr and MRL/lpr.Camkiv−/− mice (data not shown). However, at 16 wk of age, kidney damage was largely diminished in MRL/lpr.Camkiv−/− mice compared with age-matched MRL/lpr mice (Supplemental Fig. 1C). MRL/lpr.Camkiv−/− mice developed significantly less glomerular, tubulointerstitial, and perivascular

**FIGURE 3.** PDGF-BB–mediated IL6 gene transcription is inhibited in MCs from MRL/lpr.Camkiv−/− mice. A–C, A total of 400,000 MCs derived from MRL/MPJ and MRL/lpr mice was treated or not with KN-93 for 48 h and stimulated with or without PDGF-BB for 24 h. IL-6 mRNA expression was assessed by PCR (A) and quantified by densitometry of agarose gels (B, C). IL-6 levels in the supernatants were measured by ELISA. D–F, A total of 400,000 MCs derived from MRL/MPJ, MRL/lpr, and MRL/lpr.Camkiv−/− mice were stimulated or not with PDGF-BB for 24 h. IL-6 mRNA expression was assessed by PCR (D) and quantified by densitometry of agarose gels (E, F). IL-6 levels in the supernatants were measured by ELISA. G, MCs from MRL/lpr and MRL/lpr.Camkiv−/− mice were transfected with empty pGL3 luciferase vector, IL6 promoter construct (mIL6p-WT), or a mutated IL6 promoter construct with a disrupted AP-1 site (mIL6p-AP1 mut); incubated or not with PDGF-BB for 24 h; and assayed for relative promoter activity (*n = 4–5 mice/group; performed in three independent experiments). *p ≤ 0.05, **p ≤ 0.01.
lesions than did MRL/lpr mice (Fig. 1A–C). As shown in Fig. 1D, proteinuria was also robustly decreased in MRL/lpr.Camkiv−/− mice. Furthermore, MRL/lpr.Camkiv−/− mice displayed significantly lower serum titers of anti-dsDNA Abs at 8 and 16 wk of age compared with MRL/lpr mice (Fig. 1E, 1F).

Mesangial proliferation is a hallmark of lupus nephritis. Incubation of MCs from MRL/MpJ and MRL/lpr mice with PDGF-BB for 24 h upregulated protein levels of cell cycle regulators CDK-2 and cyclin D1; however, pretreatment with CaMKIV inhibitor KN-93 suppressed these levels (Supplemental Fig. 2A–C). In line with this, these proteins were downregulated in MRL/lpr.Camkiv−/− MCs in the presence or absence of PDGF-BB stimulation (Supplemental Fig. 2D–F).

MCs from MRL/MpJ and MRL/lpr mice were G1 synchronized by serum starvation for 24 h and labeled with propidium iodide, and DNA content was analyzed by flow cytometry. The percentage of G1-synchronized MCs entering the G2/M phase was higher in MRL/lpr mice compared with MRL/MpJ mice (Fig. 2A). This may indicate that MCs from MRL/lpr mice actively divide, even in the absence of exogenous stimuli, suggesting increased proliferation abilities of MRL/lpr MCs. Pretreatment with KN-93 clearly diminished the percentage of MCs entering the G2/M phase, with a concomitant increase in the percentage of MCs arrested in the G0/G1 phase in PDGF-BB–stimulated MCs from MRL/lpr mice (Fig. 2B). In addition, KN-93 pretreatment reduced the frequency of cells entering the S phase (Fig. 2B). Next, we analyzed the cell cycle status of G1-synchronized MCs from MRL/lpr.Camkiv−/− mice in the absence or presence of PDGF-BB. A large percentage of MCs from the MRL/lpr.Camkiv−/− mice was arrested at the G0/G1 phase compared with control MRL/lpr and MRL/MpJ mice (Fig. 2C). Interestingly, PDGF-BB was not able to overcome the G0/G1 block imposed by the genetic deletion of CaMKIV. These findings further supported the hypothesis that MCs from MRL/lpr mice have an intrinsic ability to proliferate without exogenous stimulation.

Mesangial IL-6 production was induced by PDGF-BB stimulation and effectively suppressed by KN-93 treatment in MRL/lpr MCs at mRNA and protein levels (Fig. 3A–C). Similarly, MRL/lpr.Camkiv−/− MCs showed decreased IL-6 expression compared with MRL/lpr and MRL/MpJ MCs upon stimulation with PDGF-BB (Fig. 3D–F). Additionally, IL-6 mRNA expression in activated splenocytes was significantly decreased in MRL/lpr.Camkiv−/− mice (Supplemental Fig. 3A).

After we demonstrated that IL-6 expression was diminished in MRL/lpr.Camkiv−/− MCs, we sought to identify possible transcriptional mechanisms underlying IL-6 expression in response to PDGF-BB stimulation. IL6 promoter activity in MRL/lpr.Camkiv−/− MCs was significantly decreased compared with MRL/lpr MCs; this effect was even more pronounced following stimulation with PDGF-BB. AP-1 has been shown to be involved in IL6 gene transcription (21). Site-directed mutagenesis of an AP-1 site within the murine IL6 promoter (located 327 bp upstream of the first ATG) limited its promoter activity significantly (Fig. 3G). Although cytoplasmic protein levels of AP-1 member c-jun were increased in MRL/lpr.Camkiv−/− MCs compared with MRL/lpr MCs, it was decreased in the nuclei of these cells (Supplementary Fig. 3B–D). DNA binding assays using nuclear MC extracts and a synthetic double-stranded oligonucleotide defining the AP-1 motif showed an increased binding of nucleoprotein from MRL/lpr MCs stimulated with PDGF-BB to this site, which was diminished when nucleoprotein lysates from MRL/lpr.Camkiv−/− MCs were used (Supplemental Fig. 2E). The binding was specific because it was competed out with the cold, unlabeled probe at 10- and 50-fold excess (Supplemental Fig. 2F).

Previously, CaMKIV was shown to contribute to decreased IL-2 production in SLE T cells, and its inhibition in lupus-prone MRL/lpr mice mitigates disease pathology by interfering with immune parameters (15, 19). Our studies in the newly developed MRL/lpr.Camkiv−/− mice clearly demonstrated that CaMKIV is important in the expression of both autoimmunity and kidney pathology. They also reveal profound effects on MC proliferation and production of IL-6, which is known to be involved in the development of glomerulonephritis (5). It is undisputed that immune complexes, autoantibodies, and autoreactive T cells are important in the instigation of lupus nephritis (1), yet little emphasis has been paid to the local kidney factors that eventually execute tissue damage. Previously, it was shown that the kallikrein genes contribute to lupus nephritis in mice and men and, therefore, factors independent of the immune system are important in the expression of kidney pathology (22). Along these lines, a congenic mouse that was derived from the lupus-prone NZM2328 mouse develops severe glomerulonephritis without breaking tolerance to nuclear Ags (23). In this study, we showed that MCs from MRL/lpr mice are able to proliferate in vitro in the absence of exogenous stimuli and that this proliferation, along with the increased IL-6 production, is significantly suppressed in the genetic absence or pharmacologic inhibition of CaMKIV. We have presented proof that CaMKIV, known to be important in the suppression of the production of IL-2, is important in MC proliferation and the expression of lupus nephritis. As such, CaMKIV represents a unique link between the immune system and the biology of MC, and its targeting may prove to be of significant clinical value as it will mitigate systemic autoimmunity and suppress kidney inflammation.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figures for manuscript by Ichinose et al. “Calcium/calmodulin-dependent protein kinase type IV is essential for mesangial cell proliferation and lupus nephritis” (11-02357-CER, revision)

Supplementary Figure 1. CaMKIV expression and kidney histopathology. (A) Messenger RNA and (B) protein expression of CaMKIV in mesangial cells obtained from MRL/MPJ, MRL/lpr and MRL/lpr.camkiv/−/− mice. (C) PAS-stained kidney sections representative of glomerular, tubular and perivascular lesions of 16-week old mice are shown. Bars indicate 50 μm (glomerular), 200 μm (tubular) and 100 μm (perivascular).

Supplementary figure 2. CaMKIV suppresses cell cycle arrest of lupus mesangial cells. (A)-(C) 200,000 MCs derived from MRL/MPJ and MRL/lpr mice were treated with or without KN-93 for 48 hrs and stimulated with or without PDGF-BB for 24 hrs. Protein levels of CDK2 and cyclin D1 were determined by immunoblotting (A). Densitometries of relative CDK2 (B) and cyclin D1 (C) expression are shown. (D)-(F) 200,000 MCs derived from MRL/lpr and MRL/lpr.Camkiv/−/− mice were stimulated with or without PDGF-BB for 24 hrs. Protein levels of CDK2 and cyclin D1 were determined by immunoblotting (D). Densitometries of relative CDK2 (E) and cyclin D1 (F) expression are shown.

Supplementary figure 3. IL-6 gene transcription is inhibited in MRL/lpr.Camkiv/−/− mice. (A) Relative IL-6 mRNA expression of unstimulated and activated splenocytes in MRL/MPJ, MRL/lpr and MRL/lpr.Camkiv/−/− mice. (B) Nuclear and cytoplasmic c-Jun/AP-1 protein levels in MCs from MRL/lpr and MRL/lpr.Camkiv/−/− mice were analyzed at different time points after PDGF-BB stimulation. Densitometries of nuclear
AP-1/H3 (C) and cytoplasmic AP-1/Actin (D) expression are shown. (E) EMSA was performed with a radiolabeled oligonucleotide harboring the AP-1 site (-327) of the mouse \textit{IL6} promoter and nuclear protein extracts from MRL/\textit{lpr} and MRL/\textit{lpr.Camkiv}^{-/-} MCs (stimulated with or without PDGF). The specific protein:DNA complex containing AP-1 is indicated by an arrow. (F) For competition assays, experiments were conducted including the unlabeled wild type (comp\textsubscript{wt}) probe at a 10- and 50- fold molar excess. The specific protein:DNA complex is indicated by an arrow (n=4-5 mice/group and performed in two or three independently replicates).
Suppl. figure 2

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