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Calcium/calmodulin-dependent protein kinase type IV is essential for mesangial cell proliferation and lupus nephritis

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

[*]Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, 02215 MA, USA.

[†]Department of Nephrology and Clinical Immunology, RWTH University of Aachen, Aachen, Germany.

Abstract

Renal involvement in systemic lupus erythematosus (SLE) 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 pharmalogical inhibition approaches we demonstrate that calcium/calmodulin-dependent protein kinase type IV (CaMKIV) which contributes to altered cytokine production in SLE patients controls spontaneous and platelet derived growth factor (PDGF)-stimulated mesangial cell proliferation and promotes IL-6 production through AP-1. Our studies identify CaMKIV as a valuable treatment target for lupus nephritis and point out the importance of local kidney factors in the expression of tissue damage which if properly targeted should enhance clinical benefit and limit toxicity.

Keywords

autoimmunity; lupus nephritis; systemic lupus erythematosus; mesangial cells; CaMKIV

INTRODUCTION

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 has been 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). Various transcription factors such as AP-1, cAMP response element (CRE)-binding protein (CREB) and nuclear factor for IL-6 expression (NF-IL-6) have been identified to control *IL6* promoter activity (6–8). Amongst 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 regulate autoimmunity and cell proliferation (12–14). A small molecule inhibitor of CaMKIV, KN-93 mitigates disease development in lupus-prone mice by suppressing

Correspondence: Dr. George C. Tsokos Department of Medicine, Division of Rheumatology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, CLS 937, Boston, MA 02115, phone: 617-735-4161, fax: 617-735-4170, gtsokos@bidmc.harvard.edu.

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cytokine production and co-stimulatory 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 not only in expression of systemic autoimmunity, but also that of local renal damage.

MATERIALS AND METHODS

Mice

Female MRL/*lpr*, *Camk*^{4tm1Tch}/*J* and MRL/*MPJ* mice were purchased from Jackson Laboratory. MRL/*lpr.Camkiv*$^{-/-}$ mice were generated on a MRL/*lpr* background. Experiments were approved by the Institutional Animal Care Committee of Beth Israel Deaconess Medical Center. Measurement of anti-dsDNA antibody 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 2 independent experiments, so that the presented data display the average from a total of 8 mice/group. Kidneys from 16-week old mice were formalin-fixed, paraffin sections were PAS-stained and renal lesions were evaluated according to previously described criteria (16, 17). Scoring was performed blindly by a nephropathologist.

Primary culture of mesangial cells (MCs)

Primary MCs were isolated according to Allam *et al.* (20) 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 3 and 7. 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 of PDGF-BB (Peprotech) for 24 h. As indicated, cells were pre-treated with KN-93 (20 μM) for 48 h prior to addition of PDGF-BB. For RNA and protein analyses, EMSAs and luciferase experiments mesangial cells were pooled from 5 mice/group and each experiment was performed in 2–3 independent replicates.

Immunoblotting

Briefly, MCs were homogenized in RIPA buffer at 4°C for 30 min. After centrifugation (14,000 rpm; 30 min; 4°C) supernatants were collected. The following polyclonal rabbit antibodies were used for 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'-CCGGAGAGGAGACTTCACAG-3' (forward) and 5'-CCAGTTTGGTAGCATCCATC -3' (reverse); CaMKIV: 5'-TCACATGGACACTGCTCAGA-3' (forward) and 5'-TGCATCTTTCTCCACCTCCT-3' (reverse). 18S rRNA primers were reported previously (15).

IL-6 ELISA

400,000 primary MCs were plated on 6-well plates and serum-starved for 24 hrs. 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 FACS Scan (BD Biosciences). Data were acquired using CellQuest software (BD Biosciences); at least 10,000 events were collected for each histogram. 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). Transient transfections were performed in primary MCs (seeded at 1×10^5 cells/well) by using 1 μg of reporter DNA, 10 ng PRTK plasmid per transfection and 2 μl of Lipofectamine 2000 (Invivogen). 24 h after transfection, cells were either incubated with or without PDGF-BB (20 ng/ml) for another 24 h. Luciferase activities in the cell lysates was measured using the Dual-Luciferase Reporter Assay System (Promega). Experiments were repeated at least four times. Values in the bar diagrams are given as mean ± S.D.

EMSAs

500,000 MCs were used for preparation of nuclear protein extracts as described before (21). A double-stranded DNA probe harboring the AP-1 site (−327) of the murine *IL6* promoter (5'- AGTGCTGAGTCACTTTTAAAG -3') was [γ-^{32}P]-ATP-radiolabeled using a T4-polynucleotide kinase. EMSA was performed as described before using 5 μg of nuclear protein and 1 μg of poly(dG)·poly(dC) per reaction. (21). 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 perfomed using Image J software (NIH). Kruskal-Wallis test was used to determine statistical significance (*$P \leq 0.05$; **$P \leq 0.01$; NS = not significant).

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* (18) (Suppl. figs. 1A/B). Total numbers of splenocytes, peripheral lymph node cells and major B and T cell compartments were not different between MRL/*lpr* and MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice (data not shown). However, at 16 weeks of age kidney damage was largely diminished in MRL/*lpr*.Ca*mkiv*$^{-/-}$ when compared to age-matched MRL/*lpr* mice (Suppl. fig. 1C). MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice developed significantly less glomerular, tubulointerstitial and perivascular lesions than MRL/*lpr* mice (Figs. 1A–C). As shown in Fig. 1D, proteinuria was also robustly decreased in MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice. Furthermore, MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice displayed significantly lower serum titers of anti-dsDNA antibodies at 8 and 16 weeks of age as compared to MRL/*lpr* mice (Figs. 1E and F).

Mesangial proliferation is a hallmark of lupus nephritis. Incubation of MCs from MRL/*MPJ* and MRL/*lpr* mice with platelet derived growth factor (PDGF)-BB for 24 hours up-regulated protein levels of cell cycle regulators CDK-2 and cyclin D1, however, pre-treatment with CaMKIV inhibitor KN-93 suppressed these levels (Suppl. figs. 2A–C). In line with this, these proteins were down-regulated in MRL/*lpr*.Ca*mkiv*$^{-/-}$ MCs in the presence or absence of PDGF-BB stimulation (Suppl. figs. 2D–F).

MCs from MRL/*MPJ* and MRL/*lpr* mice were G_1-synchronized by serum starvation for 24 h, labeled with propidium iodide and DNA content was analyzed by flow cytometry. The percentage of G_1-synchronized MCs entering the G_2/M phase was higher in MRL/*lpr* mice compared to that of 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. Pre-treatment with KN-93 clearly diminished the percentage of MCs entering the G_2/M phase with a concomitant increase in the percentage of MCs arrested in the G_0/G_1 phase in PDGF-BB-stimulated MCs from MRL/*lpr* mice (Fig. 2B). In addition, KN-93 pre-treatment reduced the frequency of cells entering the S phase (Fig. 2B). Next, we analyzed the cell cycle status of G_1-synchronized MCs from the MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice in the absence or presence of PDGF-BB. A large percentage of MCs from the MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice was arrested at the G_0/G_1 phase compared to control MRL/*lpr* and MRL/*MPJ* mice (Fig. 2C). Interestingly, PDGF-BB was not able to overcome the G_0/G_1 block imposed by the genetic deletion of *CaMKIV*. These findings further support the hypothesis that MCs from MRL/*lpr* 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 (Figs. 3A–C). Similarly, MRL/*lpr.Camkiv*$^{-/-}$ MCs showed decreased IL-6 expression as compared to MRL/*MPJ* and MRL/*lpr* MCs upon stimulation with PDGF-BB (Figs. 3D–F). Additionally, IL-6 mRNA expression in activated splenocytes was significantly decreased in MRL/*lpr*.Ca*mkiv*$^{-/-}$ mice (Suppl. 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 to MRL/*lpr* MCs and this effect was even more pronounced following stimulation with PDGF-BB. AP-1 has been shown to be involved in *IL6* gene transcription (19). 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). Whereas cytoplasmic protein levels of AP-1 member c-jun were increased in MRL/*lpr.Camkiv*$^{-/-}$ MCs compared to MRL/*lpr* MCs it was found to be decreased in the nuclei of these cells (Suppl. 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 (Suppl. fig. 2E). The binding was specific because it was competed out with the cold, unlabelled probe at a 10- and 50- fold molar excess (Suppl. 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, 20). Our studies in the newly developed MRL/*lpr.Camkiv*$^{-/-}$ mice clearly demonstrate that CaMKIV is important in the expression of both, autoimmunity and kidney pathology. It reveals 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 which 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 (21). Along these lines, a congenic mouse that was derived from the lupus-prone NZM2328 mouse develops severe glomerulonephritis without breaking tolerance to nuclear antigens (22). Here we show 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 of significant clinical value as it will mitigate systemic autoimmunity and suppress kidney inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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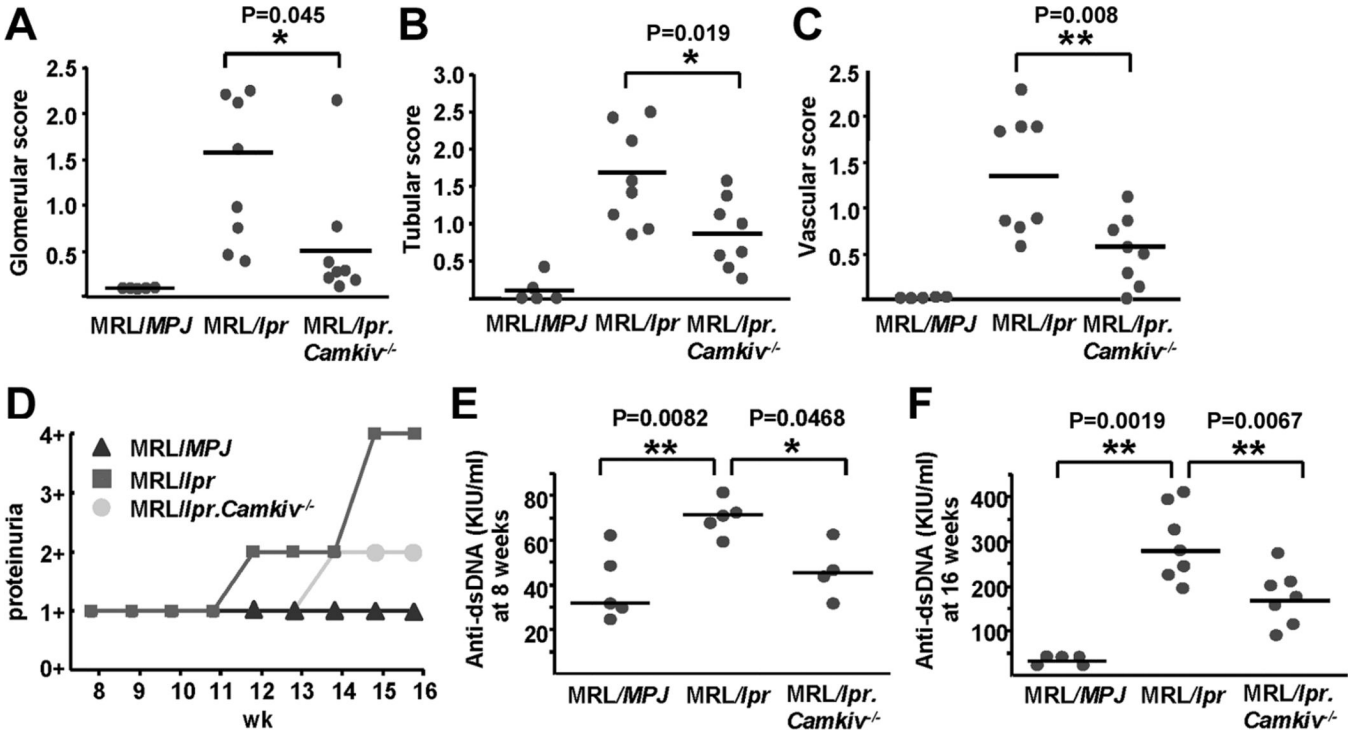

Figure 1. *CaMKIV* deficiency improves lupus pathology

(A–C) Mean scores of glomerular injury (**A**), tubular damage (**B**), and perivascular lymphocyte infiltration (**C**), from MRL/*MPJ*, MRL/*lpr* and *MRL/lpr.Camkiv*$^{-/-}$ mice are shown. (**D**) Proteinuria was quantified weekly starting from 8 weeks of age. **(E)** and **(F)** Anti-dsDNA IgG antibodies from 8-weeks old (**E**) and 16-weeks old (**F**) mice of each group were detected by ELISA (*n*=4–8 mice per group).

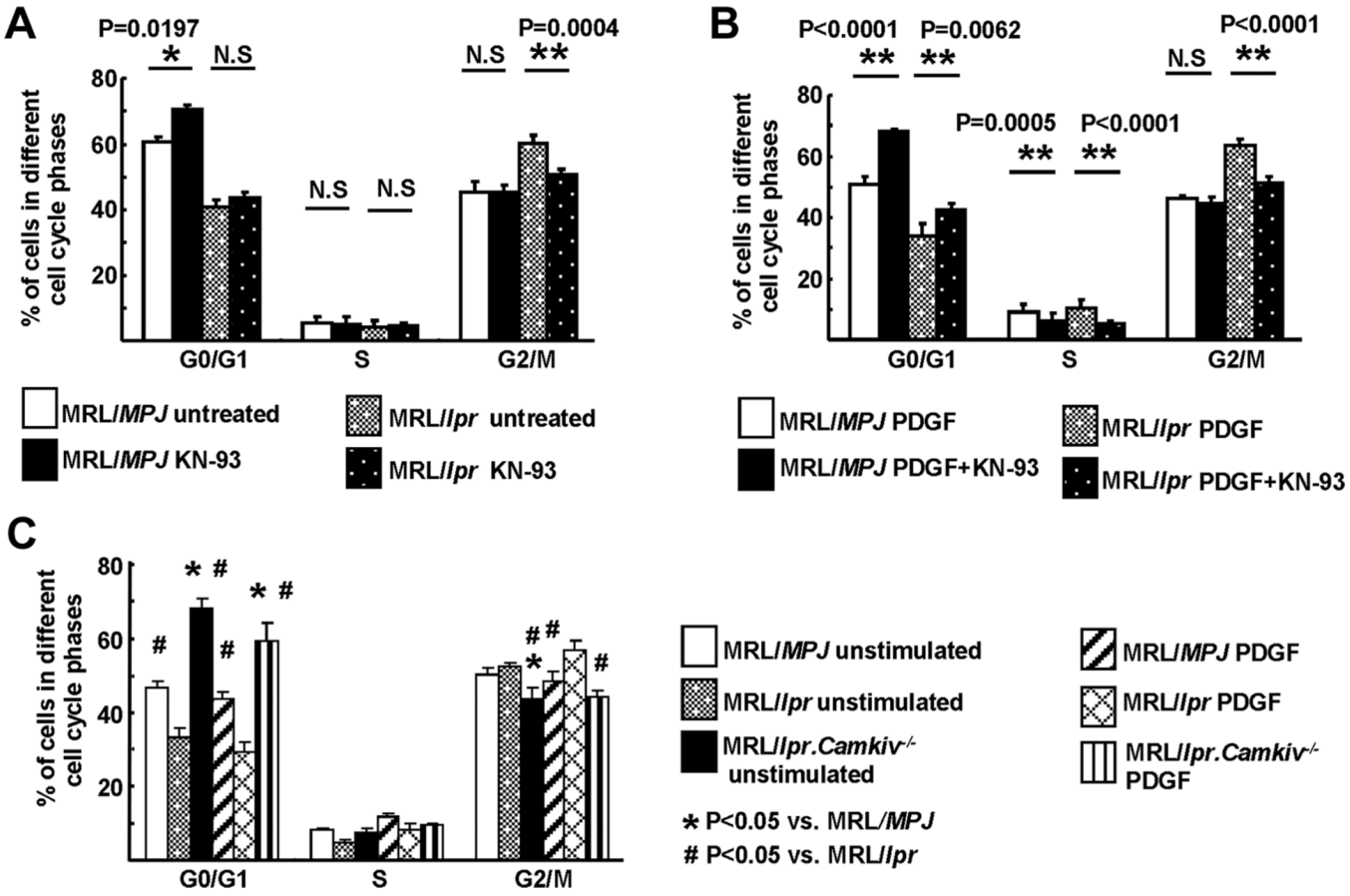

Figure 2. CaMKIV suppresses cell cycle arrest of lupus MCs

(A) and **(B)** MCs were stained by propidium iodide (PI) and cell cycle analysis was performed by flow cytometry. 400,000 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. Cumulative data of flow cytometry (percentage of cells in each cell cycle phases) are shown from **(A)** unstimulated and **(B)** PDGF-BB-stimulated MCs. Values are the mean ± SD of n=4–5 mice/group and performed in three independently replicates. **(C)** 400,000 MCs derived from MRL/*MPJ*, MRL/*lpr* and MRL/*lpr.Camkiv*$^{-/-}$ mice were stimulated with or without PDGF-BB for 24 hrs. Cumulative data of flow cytometry (percentage of cells in each cell cycle phases) are shown. Values indicate mean ± SD of n=4–5 mice/group performed in three independently replicates.

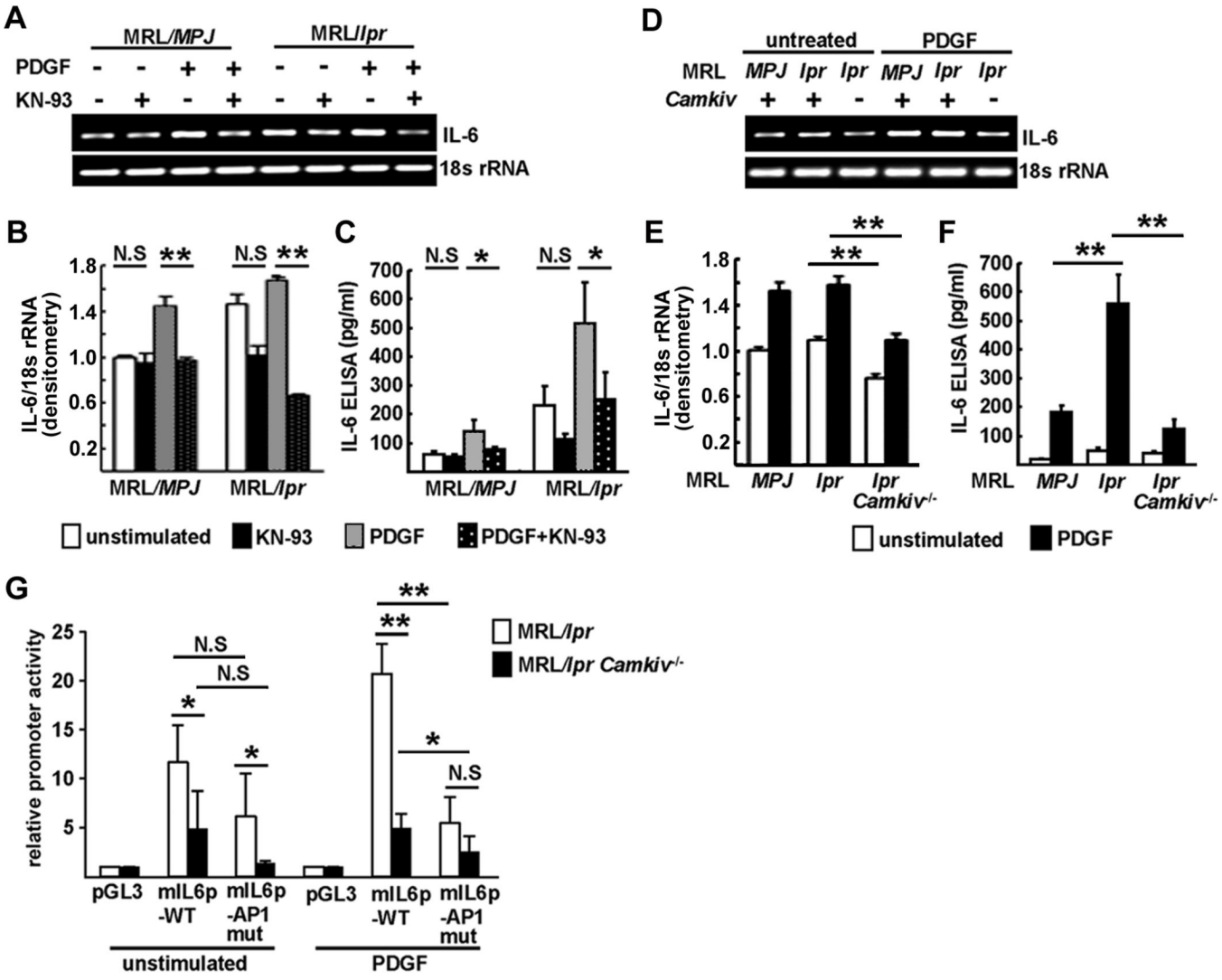

Figure 3. PDGF-BB-mediated *IL6* gene transcription is inhibited in MCs from MRL/*lpr.Camkiv*$^{-/-}$ mice

(A)–(C) 400,000 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. IL-6 mRNA expression was assessed by PCR **(A)** and quantified by densitometry of agarose gels **(B)**. IL-6 levels in the supernatants were measured by ELISA **(C)**. **(D)–(F)** 400,000 MCs derived from MRL/*MPJ*, MRL/*lpr* and MRL/*lpr.Camkiv*$^{-/-}$ mice were stimulated with or without PDGF-BB for 24 hrs. IL-6 mRNA expression was assessed by PCR **(D)** and quantified by densitometry of agarose gels **(E)**. IL-6 levels in the supernatants were measured by ELISA **(F)**. **(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 with or without PDGF-BB for 24 hrs and assayed for relative promoter activity (n=4–5 mice/group: performed in 3 independent experiments).