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KN-93, an inhibitor of calcium/calmodulin-dependent protein kinase IV, promotes generation and function of Foxp3⁺ regulatory T cells in MRL/*lpr* mice

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Abstract

Objective—Foxp3⁺ regulatory T cells (T_{reg}) are pivotal for the maintenance of peripheral tolerance and prevent development of autoimmune diseases. We have reported that calcium/calmodulin-dependent protein kinase IV (CaMK4) deficient MRL/*lpr* mice display less disease activity by promoting IL-2 production and increasing the activity of T_{reg} cells. To further define the mechanism of CaMK4 on T_{reg} cells in systemic lupus erythematosus (SLE), we used the *Foxp3*-GFP reporter mice and treated them with KN-93, an inhibitor of CaMK4.

Methods—We generated MRL/*lpr Foxp3*-GFP mice to record T_{reg} cells; stimulated naïve CD4⁺ T cells from MRL/*lpr Foxp3*-GFP mice under T_{reg} polarizing conditions in the absence or presence of KN-93; evaluated the number of GFP positive cells in lymphoid organs and examined skin and kidney pathology at 16 weeks of age. We also examined the infiltration of cells and recruitment of T_{reg} cells in the kidney.

Results—We show that culture of MRL/*lpr Foxp3*-GFP T cells in the presence of KN-93 promotes T_{reg} differentiation in a dose-dependent manner. Treatment of MRL/*lpr Foxp3*-GFP mice with KN-93 results in a significant induction of T_{reg} cells in the spleen, peripheral lymph nodes and peripheral blood and this is accompanied by decreased skin and kidney damage. Notably, KN-93 clearly diminishes the accumulation of inflammatory cells along with reciprocally increased T_{reg} cells in target organ.

Conclusion—Our results indicate that KN-93 treatment enhances the generation of T_{reg} cells *in vitro* and *in vivo* highlighting its potential therapeutic use for the treatment of human autoimmune diseases.

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Declaration of interest

The authors have declared that no conflict of interest exists.

Keywords

Calcium/calmodulin-dependent protein kinase IV; KN-93; organ damage; regulatory T cells; systemic lupus erythematosus

Introduction

Autoimmune diseases such as systemic lupus erythematosus (SLE) display quantitative [1] and/or qualitative [2] deficiencies of Foxp3⁺ regulatory T (T_{reg}) cells and this is considered to contribute to immune dysregulation and organ damage [3]. Calcium/calmodulin-dependent protein kinase IV (CaMK4) is expressed at increased levels in T cells from SLE patients [4] and lupus-prone mice [5]. We have previously demonstrated that KN-93, an inhibitor of CaMK4, ameliorates disease in lupus-prone mice [6] and that *Camk4* deficient MRL/*lpr* mice display less disease activity by promoting IL-2 production and increasing the activity of T_{reg} cells [5]. However, the effect of pharmacologic inhibition of CaMK4 on the generation and function of T_{reg} cells remains unclear. Accordingly, we generated MRL/*lpr* Foxp3-GFP reporter mice and evaluated the generation of T_{reg} cells during disease progression and the recruitment of T_{reg} cells in target organs.

We report that inhibition of CaMK4 by KN-93 mitigates lupus-like disease by restoring T_{reg} function in target organs as well as by correcting the number of T_{reg} cells in lymphoid organs.

Materials and methods

Mice

Female MRL/MpJ-*Tnfrsf6*^{*lpr*} (MRL/*lpr*) mice were purchased from the Jackson Laboratory. MRL/*lpr* Foxp3-GFP mice were generated at the Jackson Laboratory by backcrossing C57BL/6J Foxp3-GFP mice into MRL/*lpr* mice for 10 generations. MRL/*lpr* Foxp3-GFP mice were administered the CaMK4 inhibitor KN-93 (Calbiochem) by intraperitoneal injections at a dose of 0.24 mg/mouse/week of body weight, three times a week as previously reported [6]. KN-93 was initiated at 6 weeks of age (prior to the onset of disease) or at 12 weeks of age (established disease). Animals were sacrificed at the end of their 16 weeks of age. Mice were maintained in a specific pathogen free (SPF) animal facility (Beth Israel Deaconess Medical Center). Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.

In vitro T cell differentiation

Naïve CD4⁺ T cells were purified by magnetic cell sorting (CD4⁺CD62L⁺ T cell isolation kit II; MiltenyiBiotec, Auburn, CA) from the spleen and the purity of isolated T cell populations routinely exceeded 94%. Naïve T cells were stimulated 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 the presence of IL-2 (20 ng/ml; R&D Systems, Minneapolis, MN), TGF-β1 (3 ng/ml; R&D Systems), anti-IL-4 (10 µg/ml; C17.8; BioLegend, San Diego, CA) and anti-IFN-γ (10 µg/ml; XMG1.2; BioLegend) for the generation of T_{reg} cells.

Isolation of single cells

Spleen, lymph nodes, thymus and Peyer's patches were excised from mice, and single cell suspensions were obtained by teasing the organs through a nylon mesh. Isolation of single cells from the kidneys was modified from a previously described procedure [7]. In brief, perfusion of the kidneys was performed with 30 ml of pre-warmed phosphate buffered saline (PBS). Then kidneys were digested with collagenase type 4 (100 µg/ml) (Worthington Biochemical Corp., Freehold, NJ) in Hank's Balanced Salt Solution (HBSS) for 30 min (37 °C). Single cells of kidneys were isolated the same way as splenocytes and cell suspensions were subjected to density separation to eliminate the epithelial or tubular cells of the kidneys using lymphocyte separation medium (Fisher Scientific, Waltham, MA).

Flow cytometry and determination of cell number

Isolated cells were stained for flow cytometry with antibodies against CD3e (17A2, eBioscience, San Diego, CA), CD4 (GK1.5, BioLegend), CD8a (53–6.7, eBioscience) or CD45 (30-F11, eBioscience) for 30 min at 4 °C. Total cell numbers were determined by counting live cells. Absolute cell numbers were calculated on the basis of the percentage of each population.

Immunofluorescence staining

Immunofluorescence analysis on frozen spleen, skin and kidney sections was performed by staining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Life Technologies, Carlsbad, CA) and FITC-conjugated goat anti-mouse C3 (MP Biomedicals, Solon, OH) and specimens were analyzed with a Nikon Eclipse Ti confocal microscope (Nikon Instruments, Tokyo, Japan). Images were analyzed with EZ-C1 v.3.7 software (Tokyo, Japan).

Statistical analyses

Student two-tailed *t*-test and Mann–Whitney test were used. Statistical analyses were performed in GraphPad Prism 6.02 software (San Diego, CA). A level of $p < 0.05$ was considered significant.

Results

CaMK4 inhibition increased the number of T_{reg} cells in MRL/lpr Foxp3-GFP mice

We have previously shown that MRL/lpr *Camk4*^{-/-} mice exhibited high activity of T_{reg} cells resulting from restoration of IL-2 production [5]. In order to identify the effect of pharmacological inhibition of CaMK4 on T_{reg} cells *in vivo* and *in vitro*, we generated a knock-in *Foxp3*-GFP reporter mouse in the MRL/lpr background. MRL/lpr *Foxp3*-GFP mice were treated with the CaMK4 inhibitor KN-93 from 6 weeks of age, that is prior to the onset of disease, to determine whether it could prevent disease development. We also administered the drug to 12 weeks old mice (established disease) to determine whether it could mitigate disease activity. We analyzed the percentage of T_{reg} cells in peripheral blood every one to two weeks. As shown in Figure 1(A), T_{reg} cells decreased gradually in PBS-treated mice whereas they remained stable and significantly higher in mice treated with KN-93 (Figure 1A). A similar maintenance of the percentage of T_{reg} cells was recorded in

mice treated with KN-93 after the onset of the disease (Figure 1B). At the completion of the treatment protocol, we harvested lymphoid organs from treated and non-treated mice and determined the numbers of Foxp3⁺ cells. As can be seen in Figure 2(A and B), GFP positive cells were found in greater numbers in the spleen and the peripheral lymph nodes of KN-93-treated mice. Immunofluorescence analysis of spleen supported this result (Figure 2C). We next isolated naïve CD4⁺ cells from spleens of MRL/*lpr* Foxp3-GFP mice and cultured then in *in vitro* under T_{reg} polarizing conditions and found that T_{reg} cells were generated in greater percentages when KN-93 was present and this is in a dose-dependent manner (Figure 3). Thus, these results indicate that CaMK4 inhibition in MRL/*lpr* mice leads to increased number of T_{reg} cells in *in vivo* as well as *in vitro*.

Treatment of MRL/*lpr* Foxp3-GFP mice with KN-93 ameliorates organ damage

Pharmacological inhibition of CaMK4 by KN-93 or genetic ablation of *Camk4* is known to suppress disease activity of MRL/*lpr* mice [5,6,8]. To confirm this in our new MRL/*lpr* Foxp3-GFP mouse, we examined the deposition of C3 in the skin and the kidneys from MRL/*lpr* Foxp3-GFP mice treated either with PBS or KN-93 by immunofluorescence. As we expected, mice treated with PBS exhibited substantial deposition of C3 in both the skin and the kidney, whereas less C3 deposition was noted in the skin and kidneys of KN-93-treated mice. Likewise, less IgG deposition in kidneys was observed in KN-93-treated mice. These results indicate that T_{reg} induction by KN-93 treatment suppresses the recruitment of complement to the skin (Figure 4A and C) and the kidney (Figure 4B and C), ameliorates skin (Figure 4D) and the deposition of IgG in the glomeruli (Figure 4C and E) and kidney damage (not shown).

CaMK4 inhibition prevents the recruitment of inflammatory cells into kidneys of MRL/*lpr* Foxp3-GFP mice

CD4⁺ T cells and CD4⁻CD8⁻ (double negative, DN) T cells are present in the inflamed kidneys of MRL/*lpr* mice and contribute to nephritis progression [9,10]. Since T_{reg} cells have the ability to traffic to sites of inflammation such as skin or kidneys and mitigate organ damage [11,12], we sought to determine whether CaMK4 inhibition affects T cell subset distribution in the kidneys of MRL/*lpr* Foxp3-GFP mice. We noted significant decreases in the numbers of DN and CD4⁺ T cells in the kidneys of KN-93-treated MRL/*lpr* Foxp3-GFP mice (Figure 5A and B). Importantly though, the percentage of GFP positive cells in the kidneys was significantly higher in KN-93 treated mice compared to PBS treated mice (Figure 5C). These results show that CaMK4 is involved in the entry of T cells in the kidney and its inhibition enables the distribution among T cell subsets in favor of T_{reg} cells which may avert inflammation.

Discussion

In this report, we present a new Foxp3 reporter mouse in the MRL/*lpr* background which we used to demonstrate in a definitive manner that CaMK4 limits the generation of T_{reg} cells in lupus-prone mice and that its inhibition enables T_{reg} expansion and entry into target organs where they may limit the inflammatory process.

IL-2 is causally linked to the maintenance and function of T_{reg} cells [13]. We have previously reported that the deleted IL-2 production was restored by diminishing the activity of the transcriptional repressor activity of cAMP response element modulator (CREM)- α in MRL/*lpr* *Camk4*^{-/-} mice [5]. In line with these observations, pharmacologic inhibition of CaMK4 by KN-93 in MRL/*lpr* mice resulted in a significant increase of IL-2 production (data not shown). Although it is still unclear whether KN-93 can affect the stability, proliferation or survival of natural T_{reg} cells, it induced more T_{reg} cells from naïve CD4⁺ T cells and this might contribute the increased number of T_{reg} cells by the treatment of KN-93. Together current and published data suggest that KN-93 increases T_{reg} cells by correcting IL-2 production in MRL/*lpr* mice.

Although quantitative and/or qualitative deficiencies in circulating T_{reg} cells have been associated with an increased lupus disease activity [1,14,15], little is known about the role for T_{reg} cells in target organs both in terms of presence and function. Furthermore, the pathogenicity of kidney-infiltrating effector T cells and the protective effect of kidney-infiltrating T_{reg} cells have not been defined because of the difficulty to analyze organ-infiltrating T cell subsets. It has been reported that low-dose anti-CD3 antibody treatment enhanced T_{reg} cells in kidneys from B6D2F1 hybrids induced by chronic graft-versus-host disease (GVHD) resulting in amelioration of the severity of immune complex-mediated glomerulonephritis [16]. *FOXP3* expression in kidney tissues from SLE patients was reported to be comparable to that found in normal human kidneys [17]. Our current study demonstrates that KN-93 treatment results in increased percentages of T_{reg} cells in the kidneys of lupus prone mice and decreased number of infiltrating T cells indicating that the T_{reg} cells recruited to the kidney by KN-93 ameliorate lupus nephritis.

In conclusion, we have provided evidence that CaMK4 plays a central role in the suppression of T_{reg} cells and that its inhibition in lupus-prone mice enhances their generation and presence both in lymphoid organs and inflamed tissues where they may suppress the entry and/or function of effector T cells and organ damage.

Acknowledgments

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Figure 1.

CaMK4 inhibition enhanced the percentage of T_{reg} cells in the peripheral blood from MRL/lpr *Foxp3*-GFP mice. T_{reg} (GFP positive) cells in the peripheral blood were measured by flow cytometry after treatment. KN-93 was initiated from 6 weeks of age (A) or 12 weeks of age (B) (**p* < 0.05; mean ± SEM; cumulative results of three independent experiments with 3–5 mice per group).

Figure 2. CaMK4 inhibition increased the percentage of T_{reg} cells in the lymphoid organs from MRL/*lpr* *Foxp3*-GFP mice. (A) Representative flow cytometric staining profile for GFP positive cells in *in vivo* experiments at 16 weeks of age. KN-93 was initiated from 6 weeks of age. (B) Percentage of GFP positive cells in spleen, thymus, peripheral LN, mesenteric LN and Peyer's patch ($n = 4-6$). (C) Immunofluorescence staining of spleen sections for GFP (green) and DAPI (blue) expression. Scale bar = 50 μ m. (Color online.)

Figure 3.

CaMK4 inhibition enhanced T_{reg} cells differentiation from naïve CD4 T cells of MRL/*lpr* *Foxp3*-GFP mice. Representative flow cytometric staining profile for T_{reg} cells in *in vitro* experiments. Naïve T cells from MRL/*lpr* *Foxp3*-GFP mice were differentiated in T_{reg} condition in the absence or presence (4 or 10 μ M). A profile representative of six mice per group is shown (* $p < 0.05$, ** $p < 0.01$; mean $\pm$ SEM).

Figure 4.

KN-93 treatment ameliorates organ damage in MRL/*lpr* *Foxp3*-GFP mice. Deposition of C3 in the skin (A) or glomeruli (B) from MRL/*lpr* *Foxp3*-GFP mice at 16 weeks of age treated with PBS or KN-93 was detected by immunofluorescence with anti-C3 staining. Results are representative of 4–6 mice/group. Bar, 100 μ m. (C) The graph represents the C3 and the IgG deposition scores in the glomeruli of the indicated mice as detected by immunofluorescence ($n = 4–6$ /group). Data are representative of two independent experiments (* $p < 0.05$; mean $\pm$ SEM). (D) Facial skin lesion of MRL/*lpr* *Foxp3*-GFP mice treated with PBS or KN-93 starting at 6 weeks of age. (E) Deposition of IgG in the glomeruli from MRL/*lpr* *Foxp3*-GFP mice at 16 weeks of age treated with PBS or KN-93 was detected by immunofluorescence with anti-IgG staining. Results are representative of 4–6 mice/group. Bar, 100 μ m.

Figure 5.

Loss of CaMK4 prevents recruitment of inflammatory cells into kidneys of MRL/*lpr* *Foxp3*-GFP mice. (A) Representative staining profiles of cells isolated from kidneys of MRL/*lpr* *Foxp3*-GFP mice treated with PBS or KN-93. Data are representative of 6–8 mice per group. (B) Flow cytometric analysis of cells indicated as in (A), showing number of CD3⁺, CD45⁺, CD3⁺CD4⁺CD8⁺ (DN-T), CD3⁺CD4⁺ and CD3⁺CD8⁺ cells (**p* < 0.05; mean ± SEM; *n* = 6–8). (C) Representative flow cytometric staining profile for GFP positive cells in the

kidneys (* $p < 0.05$; mean $\pm$ SEM; $n = 6-8$). Data are cumulative results of two independent experiments with 3–4 mice per group.