

Blocking angiotensin-converting enzyme induces potent regulatory T cells and modulates TH1- and TH17-mediated autoimmunity

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The renin-angiotensin-aldosterone system (RAAS) is a major regulator of blood pressure. The octapeptide angiotensin II (AII) is proteolytically processed from the decapeptide AI by angiotensin-converting enzyme (ACE), and then acts via angiotensin type 1 and type 2 receptors (AT1R and AT2R). Inhibitors of ACE and antagonists of the AT1R are used in the treatment of hypertension, myocardial infarction, and stroke. We now show that the RAAS also plays a major role in autoimmunity, exemplified by multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). Using proteomics, we observed that RAAS is up-regulated in brain lesions of MS. AT1R was induced in myelin-specific CD4⁺ T cells and monocytes during autoimmune neuroinflammation. Blocking AII production with ACE inhibitors or inhibiting AII signaling with AT1R blockers suppressed autoreactive TH1 and TH17 cells and promoted antigen-specific CD4⁺FoxP3⁺ regulatory T cells (Treg cells) with inhibition of the canonical NF- κ B1 transcription factor complex and activation of the alternative NF- κ B2 pathway. Treatment with ACE inhibitors induces abundant CD4⁺FoxP3⁺ T cells with sufficient potency to reverse paralytic EAE. Modulation of the RAAS with inexpensive, safe pharmaceuticals used by millions worldwide is an attractive therapeutic strategy for application to human autoimmune diseases.

multiple sclerosis | lisinopril | FoxP3 | AT1R

The intersection between chronic inflammatory diseases like multiple sclerosis (MS) and the most common of all of the human chronic diseases, atherosclerosis, may go far beyond the root “sclerosis,” which is shared in both their names. The renin-angiotensin-aldosterone system (RAAS), well known for its effects on blood pressure (1), may be central to the inflammatory aspects of both of these diseases. Here, we describe the striking appearance of the RAAS in MS brain, and explore how its modulation with RAAS modulating drugs, commonly used to treat myocardial infarction and stroke (2) reduces TH1 and TH17 cytokines and induces powerful CD4⁺FoxP3⁺ regulatory T cells (Treg) cells capable of reversing the paralytic disease in the experimental autoimmune encephalomyelitis (EAE) model of MS.

Atherosclerosis has long been viewed as a disease caused by hyperlipidemia and endothelial dysfunction resulting in lipid deposition within artery vessel walls. However, atherosclerosis also may be viewed as a chronic autoimmune disease with adaptive immune responses to atherosclerosis-associated antigens including heat shock protein 60 (HSP60), oxidized low-density lipoprotein (oxLDL), and angiotensin-converting enzyme (ACE) itself (3). Intralésional TH1-polarized CD4⁺ cells contribute to the pathogenesis of atherosclerosis at early stages of the disease (4). This hallmark is shared by inflammatory diseases such as rheumatoid arthritis and MS, where TH1-type cytokines like tumor necrosis factor (TNF) and IFN- γ (IFN- γ) play key pathogenic roles in these two diseases, respectively (5). Moreover, endogenous Treg cells suppress the pathogenic TH1

response in classical inflammatory diseases (6) and in atherosclerosis (7). AT1R-expressing T cells may be crucial for promoting hypertension, vascular inflammation, and atherosclerosis (8).

Here we addressed the role of angiotensin II in differentiation and function of antigen-specific TH1 and TH17 cells. We analyzed the function of AT1R in EAE, a model of multiple sclerosis where both TH1 and TH17 are critical in pathogenesis (9), and we combined this with observations on the expression of the angiotensin pathway in brain lesions of MS itself using proteomics and immunohistochemistry on autopsied human brain tissue from cases of MS.

Results

Proteomic analysis of MS plaques (10) revealed that peptides related to the RAAS system are present in CNS lesions of MS patients (Table S1 and Fig. S1). Next, the transcriptional profile of the RAAS related proteins angiotensinogen (Ang), ACE, and AT1R was analyzed in T cells from mice immunized with the encephalitogenic proteolipoprotein (PLP) peptide PLP139–151, to induce EAE. Immunization with PLP139–151 induced strong expression of AT1R in lymph node cells (LNC) (Fig. 1A and B) whereas expression of Ang and ACE remained unchanged (Fig. S2), indicating that AT1R is activated in antigen-specific T cells during the peripheral immune response to autoantigen. In addition, AII binding was increased in PLP-activated CD4⁺ T cells (Fig. 1C) and to a lesser extend in activated CD11b⁺ monocytes (Fig. S2). Moreover, AT1R expression was up-regulated in spinal cord tissue of mice with EAE (Fig. 1D–F) whereas ACE mRNA levels remained unchanged and Ang was down-regulated (Fig. S2).

AT1R was strongly up-regulated in infiltrates of plaques from the brains of MS patients as revealed by immunohistochemistry (Fig. 2A–J). Of note, AT1R expression in active MS plaques was mostly present in perivascular cuffs (Fig. 2A–F), whereas in inactive plaques, AT1R was expressed in endothelial cells, astrocytes, and axons (Fig. 2G–J and Fig. S2). These same plaques from MS patients were the subject of a previous large-scale proteomic analysis of defined MS lesions (10). From these studies one can conclude that the presence of key elements

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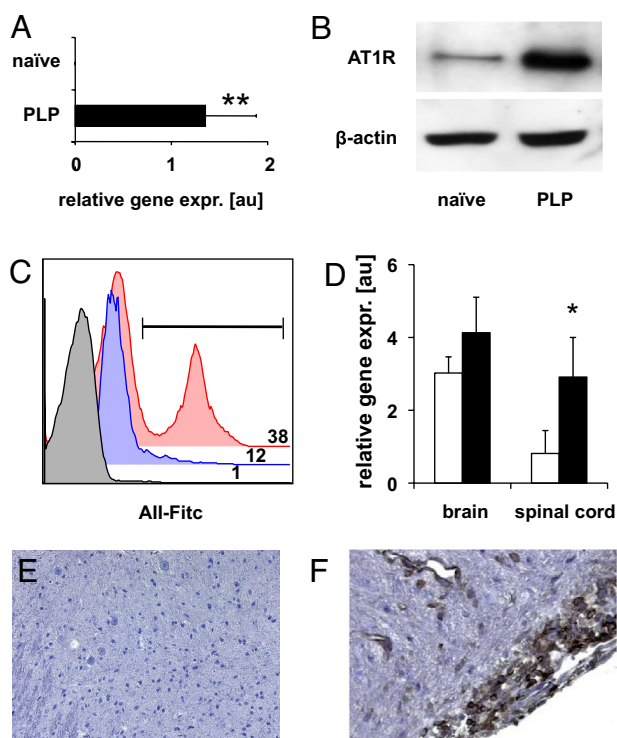


Fig. 1. Expression of AT1R during autoimmune neuroinflammation and MS. (A) Analysis of mRNA expression of AT1R in LNC from naive SJL/J mice (open bars) and SJL/J mice 10 days after immunization with PLP p139–151 (PLP, closed bars) ($n = 3$) using real-time PCR. Values represent mean arbitrary expression levels of triplicates and SEM normalized to expression of β -actin. *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis of AT1R protein expression in splenic CD4⁺ T cells from naive SJL/J mice and SJL/J mice 10 days after immunization with PLP p139–151 (PLP). (C) Binding of FITC-labeled All to myelin-specific CD4⁺ T cells as measured by flow cytometry. Splenocytes were isolated from SJL/J mice 5 days after immunization with PLP p139–151 (red histogram) or naive mice (blue histogram). Splenocytes were restimulated ex vivo with PLP p139–151 and CD4⁺ T cells were gated. Unlabelled All served as a control (gray histograms). Numbers indicate percent All binding cells. (D) Analysis of mRNA expression of AT1R in CNS tissue from naive SJL/J mice (open bars) and SJL/J mice 14 days after immunization with PLP p139–151 (PLP, closed bars) ($n = 5$) using real-time PCR. Values represent mean arbitrary expression levels of triplicates and SEM normalized to expression of β -actin. *, $P < 0.05$. (E and F) Immunohistochemical analysis of AT1R expression in meningeal infiltrates of spinal cords from naive SJL/J mice (E) and SJL/J mice 14 days after PLP-immunization (F). Magnifications: 40× (E) and 60× (F).

of the RAAS is present at the site of disease in MS, not only on immune cells but also on neurons and glia.

We asked whether activation of the AT1R in T cells might influence the proinflammatory TH1 and TH17 response to myelin antigens in EAE. Immunization with PLP139–151 led to an induction of AII in CD4⁺ T cells, CD11b⁺ monocytes and to an increase of serum AII levels (Fig. 3A), demonstrating that the RAAS system is activated in peripheral immune cells. Pretreatment of mice, immunized with PLP139–151, with the ACE inhibitor lisinopril {N2-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline} or the AT1R antagonist candesartan (3-[[2'-(2H-tetrazol-5-yl)biphenyl-4-yl]methyl]-2-ethoxy-3H-benzo[d]imidazole-4-carboxylic acid) resulted in suppression of TH1 and TH17 cytokine release and up-regulation of immunosuppressive cytokines such as IL-10 and transforming growth factor- β (TGF- β) (Fig. 3B and Fig. S3A). TGF- β is a Janus-like cytokine, that depending on context, are either proinflammatory or antiinflammatory (11). PLP-activated TH cells from mice immunized with PLP p139–151 treated with lisinopril failed to induce

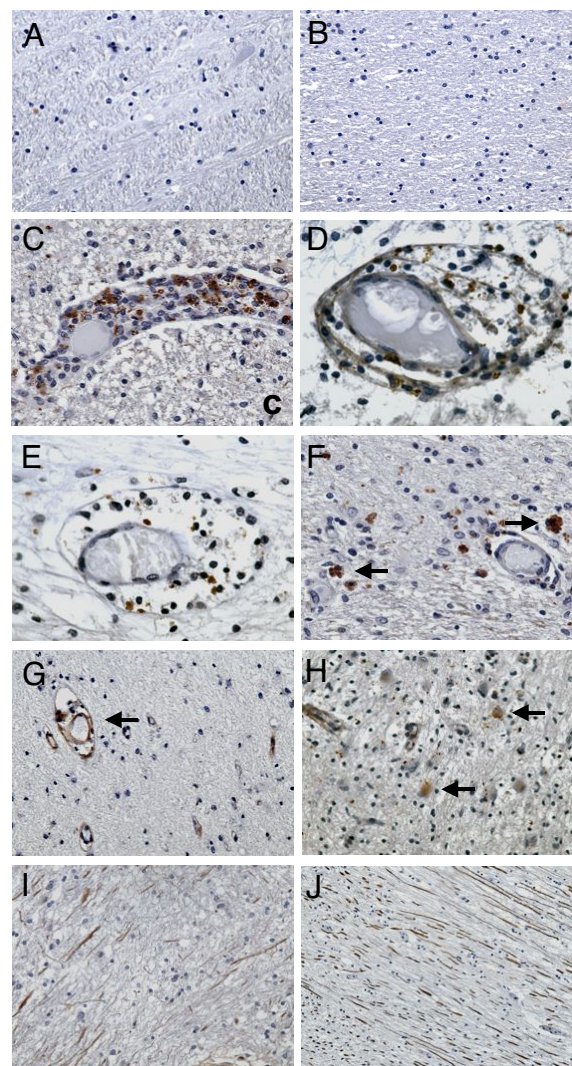


Fig. 2. Expression of AT1R in MS plaques. Immunohistochemical analysis of AT1R in human CNS tissue. No AT1R expression is detected in normal spinal cord (A) or white matter from a patient with Alzheimer disease (B). Strong AT1R expression is detected in perivascular cuffs of a chronic active MS plaque (C–F), particularly in foamy macrophages (F, arrows). CD3 staining (E) of a neighbouring section of D shows presence of T cells. AT1R is also detectable in endothelial cells (G, arrow), astrocytes (H, arrows), and axons (I) within a chronic inactive MS plaque. AT1R is also strongly expressed in axons during viral encephalitis (J), suggesting that inflammation itself may drive neuronal AT1R expression. Magnifications: 20× (J), 40× (A and I), 60× (C, F–H), and 90× (D and E).

disease in recipient mice after adoptive transfer (Fig. 3C), indicating that suppressing AII production or blockade of signals through the AT1R abolished the neuroinflammatory phenotype of myelin-specific TH cells.

Importantly, treatment with lisinopril induced the expression of FoxP3 in CD4⁺CD25⁺ T cells (Fig. 4A) and the phosphorylation of SMAD 2/3 (Fig. 4B) in CD3⁺ T cells. Thus, exposing antigen-specific T cells to its cognate antigen in the presence of lisinopril induces FoxP3⁺ regulatory T cells. Adoptive transfer of lisinopril-treated PLP-activated CD4⁺ T cells protected PLP-immunized mice from severe clinical signs of EAE (Fig. 4C). Suppression of the neuroinflammatory phenotype of PLP-activated TH cells by blocking AT1R signals is mediated through T cells directly because siRNA-mediated suppression of AT1R in CD4⁺ T cells led to a reduction of IL-17 and IFN- γ production

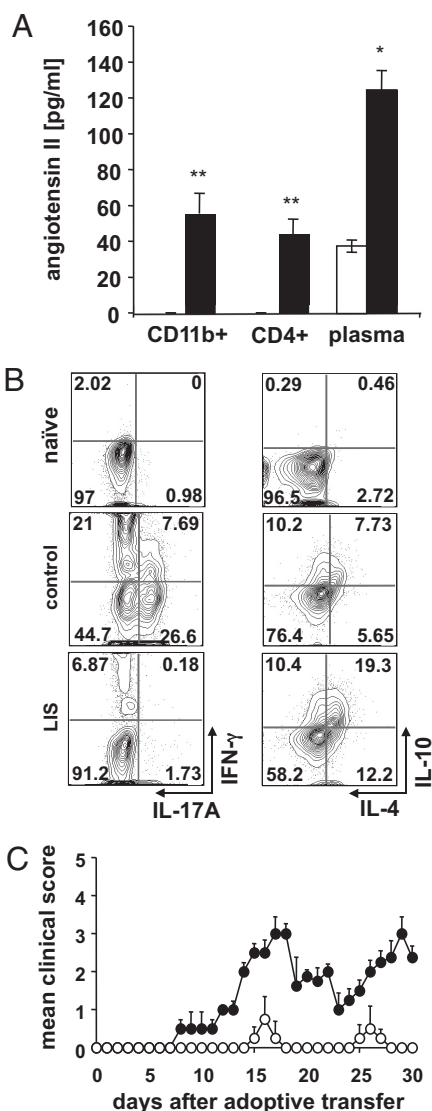


Fig. 3. Suppression of myelin-specific TH1 and TH17 cells by blocking AII production. (A) Analysis of AII in serum and in the supernatant of CD11b+ monocytes and CD4+ T cells of naïve (white bars) or SJL/J mice 10 days after immunization with PLP p139–151 (black bars) by ELISA. Cells were purified to 95–99% using CD4 MACS beads and were then incubated for 12 h with full stimulation media and supernatants were harvested. *, $P < 0.05$; **, $P < 0.01$. (B) Intracellular cytokine analysis using flow cytometry in CD4+ LNC from naïve SJL/J mice or SJL/J mice 10 days after immunization with PLP p139–151 treated with vehicle (control) or lisinopril (LIS) at 10 mg/kg/day ($n = 3$). LNC were isolated and restimulated with α CD3/ α CD28 and pulsed with PMA, ionomycin, and golgi stop. Numbers indicate percent positive cells. (C) Adoptive transfer of CD4+ LNC from SJL/J mice immunized with PLP p139–151 and treated with vehicle (filled circles) or lisinopril at 10 mg/kg/day (open circles) for 12 days. Cells were transferred into naïve SJL/J recipient mice. Data represent clinical scores as described in the *Materials and Methods* section.

whereas exogenous expression of AT1R increased the production of these cytokines (Fig. 4D and Fig. S3B). AT1R signaling, however, also influences the phenotype of antigen presenting cells because AT1R is expressed on CD11b+ monocytes and siRNA-mediated suppression of AT1R down-regulates the production of proinflammatory cytokines in these cells (Fig. S3C).

To make these studies relevant to translation into humans with MS, we chose doses appropriately scaled for surface area of the mice, based on human doses, following guidances from the US Food and Drug Administration (<http://www.fda.gov/cber/gdlns/>

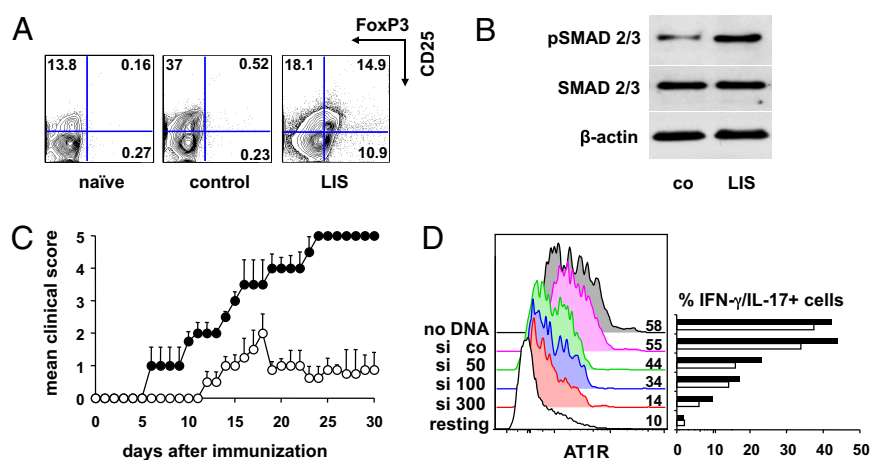
dose.pdf). These doses are similar to equivalent human doses in current use for the in vivo experiments. When lisinopril was given in the same mg/kg range as human doses, similar effects were observed (Fig. S4). Thus, we cover a range of doses for lisinopril, that are within the range of equivalent human doses by two commonly used metrics, including guidance provided by the FDA. Collectively these data indicate that modulation of production of AII via inhibition of angiotensin converting enzyme with lisinopril, leading to diminished activation of AT1R, induces a regulatory phenotype capable of suppressing TH1/TH17-mediated immune responses to autoantigens.

Next we investigated which signaling events are involved in AT1R-mediated regulation of T-cell differentiation. Activation of AT1R triggers multiple G protein-dependent and -independent signaling pathways resulting in the activation of STAT molecules. The Janus kinase (JAK)-STAT pathway is critically involved in transducing cytokine receptor signals in T cells, thus influencing T-cell differentiation (12). Thus, we tested the hypothesis that STAT molecules might be involved in the induction of regulatory FoxP3+ cells mediated by blocking AT1R signaling in antigen-specific T cells. Treatment of PLP139–151-immunized SJL/J mice with lisinopril resulted in a profound reduction of total STAT-1 and STAT-4 protein levels in PLP-specific TH cells (Fig. 5A). Moreover, LNC from lisinopril-treated animals displayed reduced responsiveness to IL-12, further indicating suppression of signaling through the STAT-4 pathway (Fig. 5B). STAT4 is critically involved in the differentiation of TH1 (13) and TH17 (14) cells and STAT4-deficient mice are resistant to EAE (15). In contrast, STAT-1-deficient mice display an increased susceptibility to EAE (16), indicating that complete ablation of STAT1 results in defective IFN- γ -mediated elimination of autoaggressive TH cells. Although these changes might simply reflect the alteration of the cytokine profile induced by lisinopril with reduction of IFN- γ , IL-12, and induction of IL-10, we observed an induction of the suppressors of cytokine signaling-1 and -3 (SOCS-1 and SOCS-3), two negative regulators of the JAK-STAT pathway (Fig. 5C). SOCS-1 is a powerful suppressor of proinflammatory cytokine signaling and required for the differentiation of CD4+ T cells (17). SOCS-1-deficient mice develop severe multiorgan inflammatory disease (18) and treatment with a SOCS-1-mimetic peptide protected mice from EAE (19).

Collectively, our data show the impact of reduction of signals through AT1R, via diminished production of AII after ACE blockade: lisinopril treatment of antigen-specific T cells interferes with cytokine signaling to induce a regulatory phenotype. Because SOCS-1 negatively regulates NF- κ B (20) and the proinflammatory effects of AII have been attributed to the activation of NF- κ B, we tested the hypothesis that NF- κ B is involved in the induction of Treg cells mediated by blocking AT1R signals in antigen-specific T cells. Treatment of PLP-immunized mice with lisinopril suppressed the expression and DNA binding of p65 (RelA) and c-rel while inducing the expression and DNA binding of inhibitory κ B α (I κ B α) and Relb in antigen-specific T cells (Fig. 5D and E and Fig. S5A), thus suppressing the canonical NF- κ B pathway while inducing the alternative NF- κ B pathway. These data are concordant with studies in transgenic mouse models showing that both NF- κ B1 (p50)- and c-Rel-deficient mice are resistant to EAE (21) whereas RelB-deficient mice displayed exaggerated inflammatory responses with increased expression of TH1 cytokines (22). Again, skewing of the NF- κ B pathway by lisinopril was not unique to T cells but was also observed in monocytes (Fig. S5B). Collectively, our results support the notion that the intrinsic classical NF- κ B pathway is important for TH1 differentiation.

Suppression of antigen-specific TH1 and TH17 responses and induction of antigen-specific Treg cells has been targeted as a

Fig. 4. Induction of myelin-specific FoxP3⁺ regulatory T cells by blocking AII production. (A) Flow cytometric analysis of FoxP3 and CD25 in CD4⁺ LNC from naïve SJL/J mice (Left) or SJL/J mice 10 days after immunization with PLP p139–151 treated with vehicle (Center, control) or lisinopril (Right, LIS) at 10 mg/kg/day ($n = 3$). Numbers indicate percent positive cells. (B) Western blot analysis of SMAD 2/3 phosphorylation in CD3⁺ LNC from SJL/J mice 10 days after immunization with PLP p139–151 treated with vehicle (co) or lisinopril (LIS) at 10 mg/kg/day ($n = 3$). (C) Adoptive transfer of CD4⁺ LNC from SJL/J mice ($n = 5$) immunized with PLP p139–151 to induce EAE and treated with vehicle (filled circles) or lisinopril at 10 mg/kg/day (open circles) for 12 days. Cells were transferred into SJL/J recipient mice ($n = 10$ per group). Recipient mice were immunized with PLP p139–151 24 h after the adoptive transfer. Data represent clinical scores as described in the *Materials and Methods* section. (D) Intracellular expression of IFN- γ (black bars) and IL-17 (white bars) using flow cytometry in CD4⁺ T cells from naïve 2D2 mice, transfected with AT1R siRNA at different concentrations using nucleofection and activated with MOG35–55 for 48 h. Histograms represent AT1R cell surface expression, numbers represent percent AT1R-positive cells. Values in the bar graphs represent percent IFN- γ or IL17⁺ cells.



therapeutic strategy to treat TH1-mediated autoimmune diseases. We thus analyzed whether inhibition of AII production or blocking AT1R suppresses TH1/TH17-mediated autoimmunity.

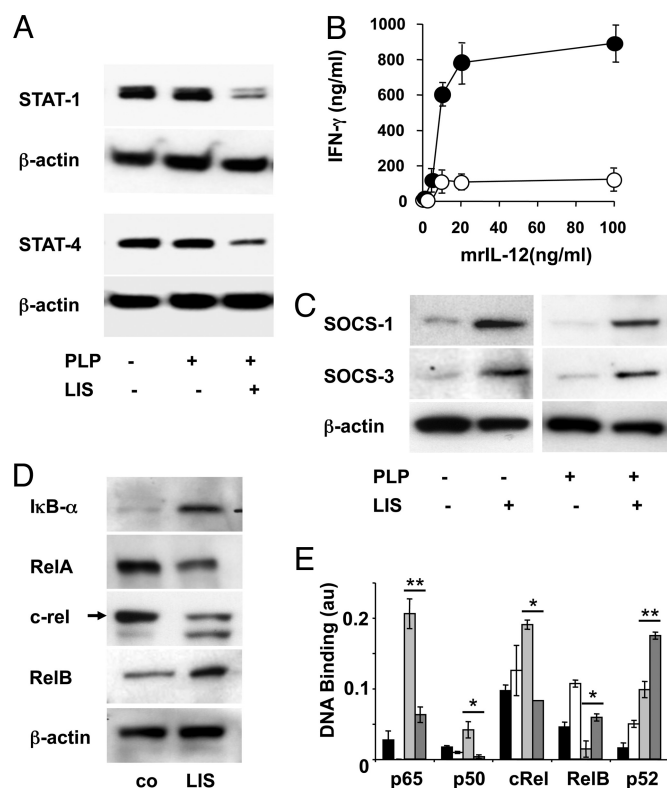


Fig. 5. Molecular mechanisms of tolerance induction by suppressing signaling through AT1R. (A, C, and D) Western blot analyses of STAT protein expression (A), SOCS protein expression (C) and expression of proteins of the NF- κ B signaling pathway (D) in CD4⁺ T cells from naïve SJL/J mice or SJL/J mice 10 days after PLP immunization with or without treatment with lisinopril. (B) IFN- γ release from CD4⁺ T cells from untreated SJL/J mice (black circles) or SJL/J mice treated with lisinopril for 10 days (open circles) after stimulation with IL-12 in vitro. (E) In vitro DNA binding studies of proteins from the NF- κ B signaling pathway in CD4⁺ T cells from naïve untreated (black bars), naïve lisinopril-treated (white bars), PLP-immunized untreated (light gray bars) and PLP-immunized lisinopril-treated SJL/J mice (dark gray bars). Values represent mean \pm SEM of arbitrary units of colorimetric analysis of DNA-binding activity. *, $P < 0.05$; **, $P < 0.01$.

Treatment of PLP-immunized mice with lisinopril prevented signs of EAE when administered before immunization (Fig. 6A), and reversed paralysis when administered during established EAE (Fig. 6B). The AT1R antagonist candesartan was equally effective in reversing paralysis in EAE, indicating that reduction in signaling through the AT1R is critical for the therapeutic effect of the ACE inhibitor lisinopril (Fig. 6C). Histopathology of CNS tissue from treated mice with EAE revealed that the therapeutic effects of lisinopril and candesartan were associated with a reduction of inflammatory foci (Fig. 6D and E) and an increase in FoxP3⁺ Treg cells in the CNS (Fig. 6F). These data indicate that reducing signals through AT1R ameliorates EAE, a paradigmatic TH1/TH17-mediated autoimmune disease model by inducing potent Treg cells.

Discussion

Earlier published studies showed that treatment with the ACE inhibitor captopril prevents EAE in Lewis rats, although disease reversal was never attempted in that work done at a time when TH17 and Treg cells had not yet been discovered (23). Administration of AT1R blockers suppressed antigen-specific T-cell responses and prevented and reversed collagen-induced arthritis (24, 25). The mechanism of how AT1R suppresses TH-mediated autoimmunity was not elucidated in these two earlier studies.

One of the major regulators of blood pressure, ACE, may play an important role in both organ-specific autoimmunity and in atherosclerosis. For instance, in MS, a polymorphism in the ACE gene is associated with susceptibility to disease, and increased ACE activity in the serum and cerebrospinal fluid (CSF) of patients with MS may be an indicator of inflammatory activity (26, 27). The results of these experiments and our study imply that the RAAS system may be critically involved in promoting TH1/TH17-mediated autoimmune diseases such as multiple sclerosis. These observations are supported by studies in cardiovascular diseases showing that AII stimulates IFN- γ and TNF in peripheral T cells in an animal model of hypertension (8) and that endogenous AII elicits a TH1 response in apolipoprotein E (ApoE)-deficient mice (28).

The first ACE inhibitor, lisinopril, is now a generic drug used by millions across the globe. Lisinopril was first designed by a simple "paper and pencil" model of substrate and inhibitor binding to the enzyme (29), and ultimately mapped at 2 Å with an X-ray crystal structure showing how it binds to human ACE (30). ACE inhibitors afford the opportunity when given in routine therapeutic doses to raise large numbers of antigen

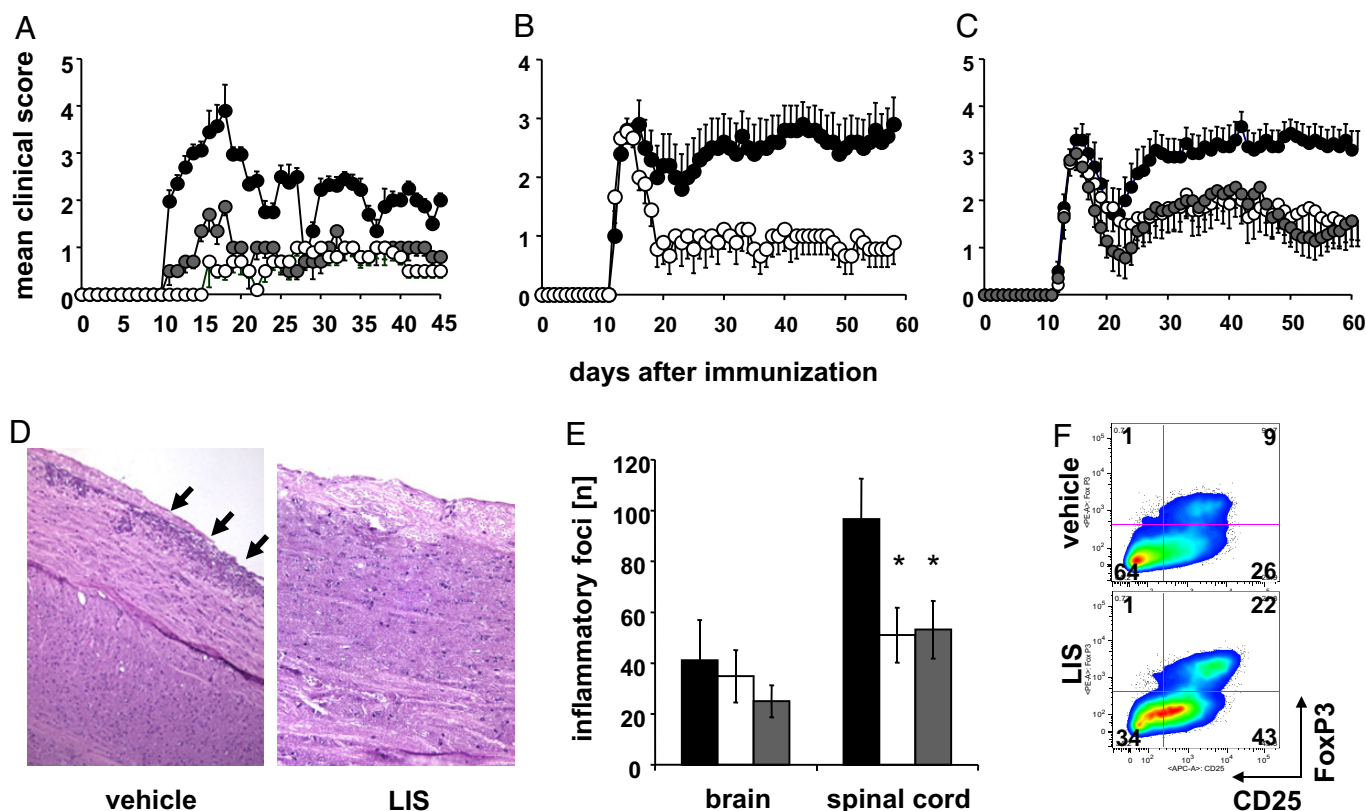


Fig. 6. Modulation of EAE by suppressing AII production or blocking AT1R. (A) Prevention of EAE after PLP immunization by lisinopril at 1 mg/kg/day (gray circles) or 10 mg/kg/day (open circles) compared with vehicle-treated controls (black circles), $n = 12$ per group. Treatment was initiated 2 days before immunization. Values are displayed as mean clinical scores as in Fig. 3. (B) Treatment of EAE after PLP immunization with lisinopril at 10 mg/kg/day (open circles) with vehicle controls (black circles), $n = 15$ per group. Treatment was initiated at the peak of first clinical disease activity (day 15 after immunization). Values are displayed as mean clinical scores. (C) Treatment of EAE after PLP immunization with lisinopril at 10 mg/kg/day (open circles) or candesartan at 1 mg/kg/day (gray circles) compared with vehicle controls (black circles), $n = 15$ per group. Treatment was initiated at the peak of first clinical disease activity (day 15 after immunization). Values are displayed as mean clinical scores. (D) H&E stained spinal cord sections of SJL/J mice with EAE with (EAE + LIS) or without (vehicle) treatment with lisinopril. Arrows indicate meningeal inflammatory infiltrate. (Magnification: D, $20\times$.) (E) Number of inflammatory foci (\pm SEM) in brains and spinal cords of SJL/J mice treated with vehicle (black bars), lisinopril (white bars), or candesartan (gray bars). Lesions were counted by a neuropathologist blinded to the type of treatment, $n = 6$ per group. *, $P < 0.05$. (F) Flow cytometric analysis of FoxP3 and CD25 in CD4⁺ CNS-infiltrating T cells from SJL/J mice with EAE 14 days after immunization with PLP139–151 treated with vehicle (Top) or lisinopril at 10 mg/kg/day (LIS, Bottom) ($n = 3$). Numbers indicate percent positive cells.

specific Treg cells with strong clinical efficacy. Early clinical data indeed indicate that ACE inhibitor therapy is associated with decreased TH1/TH2 cytokine ratios and inflammatory cytokine production in patients with chronic heart failure (31). Larger studies on Th1/Th2/Th17 and Treg cells must be undertaken in individuals with MS and in individuals being treated with these drugs for hypertension. ACE inhibitors ought to be tested clinically in MS and in other autoimmune diseases where potent antigen specific Treg cells may be a suitable treatment strategy.

Materials and Methods

EAE was induced in female SJL/J mice (8–12 weeks) via s.c. immunization with PLP p139–151 emulsified in complete Freund's adjuvans or by adoptive transfer injecting 5×10^6 T cells intravenously into naive SJL/J recipient mice. Mice ($n = 10$ –15 per treatment group) were examined daily for clinical signs of EAE and were scored as followed: 0, no clinical disease; 1, tail weakness or paralysis; 2, hindlimb weakness or paralysis; 3, complete hindlimb paralysis and forelimb weakness; 4, hindlimb paralysis and forelimb paralysis; 5, moribund or dead. Cytokine and AII levels were determined by using specific ELISA. For intracellular cytokine analysis CD4⁺ T cells or CD11b⁺ monocytes cells were fixed and permeabilized with cytofix/cytoperm and perm/wash buffers. For RT-PCR-experiments total RNA was isolated from LNC or CD3⁺ splenic T cells or brains or spinal cords. ACE, angiotensinogen (Ang), angiotensin II type 1 receptor (AT1R), and β -actin cDNAs were amplified using a Lightcycler (Roche). Trans-

location of NF κ B isoforms into the nucleus was tested using nuclear extracts isolated from CD4⁺ T cells treated with Lisinopril or PBS. For AT1R siRNA experiments CD4⁺ T cells or CD11b⁺ monocytes were transfected with different concentrations of siRNA, or control siRNA using a nucleofactor electroporation system and either a mouse primary T-cell nucleofactor kit or a mouse primary macrophage kit. For histopathology and immunohistochemistry anesthetized mice were perfused with 20 mL cold PBS followed by 20 mL of 4% (wt/vol) paraformaldehyde for fixation. Selected brain, thoracic, and lumbar spinal cord sections were evaluated by an examiner blinded to the treatment status of the animal. For AT1R expression cryostat sections (4–6 mm) were fixed with acetone and then labeled with anti-AT1R using the avidin-biotin technique. Staining was visualized by reaction with diaminobenzidine (DAB). Slides were counterstained with hematoxylin. MS and control brain samples were analyzed as described in ref. 10. Data are presented as mean \pm SEM. For clinical scores, significance between each two groups was examined by using the Mann-Whitney U test. All other statistics were analyzed by using a one-way multiple-range ANOVA test for multiple comparisons. $P \leq 0.05$ was considered significant.

See *SI Text* for full methods.

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