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Kv1.3 channels are a therapeutic target for T cell-mediated autoimmune diseases

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Autoreactive memory T lymphocytes are implicated in the pathogenesis of autoimmune diseases. Here we demonstrate that disease-associated autoreactive T cells from patients with type-1 diabetes mellitus or rheumatoid arthritis (RA) are mainly $CD4^+CCR7^-CD45RA^-$ effector memory T cells (T_{EM} cells) with elevated Kv1.3 potassium channel expression. In contrast, T cells with other antigen specificities from these patients, or autoreactive T cells from healthy individuals and disease controls, express low levels of Kv1.3 and are predominantly naïve or central-memory (T_{CM}) cells. In T_{EM} cells, Kv1.3 traffics to the immunological synapse during antigen presentation where it colocalizes with Kvβ2, SAP97, ZIP, $p56^{lck}$, and CD4. Although Kv1.3 inhibitors [ShK(L5)-amide (SL5) and PAP1] do not prevent immunological synapse formation, they suppress Ca^{2+}-signaling, cytokine production, and proliferation of autoantigen-specific T_{EM} cells at pharmacologically relevant concentrations while sparing other classes of T cells. Kv1.3 inhibitors ameliorate pristane-induced arthritis in rats and reduce the incidence of experimental autoimmune diabetes in diabetes-prone (DP-BB/W) rats. Repeated dosing with Kv1.3 inhibitors in rats has not revealed systemic toxicity. Further development of Kv1.3 blockers for autoimmune disease therapy is warranted.

effector memory T cell | rheumatoid arthritis | type-1 diabetes mellitus

T cell-mediated autoimmune diseases afflict millions of people. Autoantigen-specific therapies would be ideal. Vaccination trials with altered myelin-peptide ligand induced a nonencephalitogenic T helper 2 response in some multiple sclerosis (MS) patients and worsened disease in others (1, 2). Disease-modifying immunotherapies have improved the management of autoimmune diseases; however, each of these therapies is known to induce side effects (3–7). Consequently, there is an unmet medical need for novel immunomodulators with different mechanisms of action and/or adverse-effect profiles from existing drugs. Although the frequency of autoreactive T cells in healthy individuals and in patients with autoimmune diseases is similar (8, 9), disease-associated autoreactive T cells are mainly costimulation-independent $CCR7^-$ T_{EM} cells, whereas autoreactive T cells in healthy individuals are naïve/T_{CM} cells (10–16). Therapies that selectively suppress T_{EM} cells without affecting other lymphoid subsets would have immense value.

Kv1.3 is one of 76 human K^+ channel genes, and the homotetrameric Kv1.3 channel in T cells has distinct biophysical and pharmacological properties (17). Kv1.3 regulates membrane potential and Ca^{2+} signaling in human T cells, and its expression is increased 4- to 5-fold in activated $CD4^+$ and $CD8^+$ T_{EM}/T_{EMRA} cells. In contrast, human naïve or T_{CM} cells up-regulate the calcium-activated KCa3.1 channel to regulate membrane potential and Ca^{2+} signaling in the activated state (10). We previously showed that myelin-specific $CD4^+$ T cells from the peripheral blood (PB) of MS patients and T cells in MS lesions in postmortem brain sections were $CCR7^-CD45RA^-$ T_{EM} cells with elevated Kv1.3 levels (10, 13). Furthermore, 5-methoxypsoralen, an analog of the Kv1.3 inhibitor PAP1, ameliorated visual field defects, spasticity, and paraparesis in MS patients (18), and selective *in vivo* Kv1.3 inhibition ameliorated disease in a rat model for MS induced by myelin-specific $CD4^+CD45RC^-$ memory T cells (19, 20). In the present study we directly assayed disease-associated autoreactive T cells from patients with rheumatoid arthritis (RA) or type-1 diabetes mellitus (T1DM), and we tested whether selective Kv1.3 blockers (20, 21) alleviated autoimmune-mediated disease in rat models of RA or T1DM without causing toxicity.

Results and Discussion

Disease-Associated Autoreactive T Cells from Patients with RA or T1DM Are $CCR7^-$ $Kv1.3^{high}$ T_{EM} Cells. We measured Kv1.3 currents in T cells from synovial fluid (SF) and PB of RA or nonautoimmune osteoarthritis (OA) patients (Table 1, which is published as supporting information on the PNAS web site). Activated T cells were patch-clamped 48 h after stimulation with anti-CD3 Ab. RA-SF-T cells displayed higher numbers of Kv1.3 channels compared with OA-SF-T cells ($P < 0.0001$) (Fig. 1*A* and Table 2, which is published as supporting information on the

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Conflict of interest statement: C.B., H.W., M.W.P., G.A.G., and K.G.C. helped found a company, AIRMID, with the hope of developing Kv1.3 inhibitors as therapeutics for autoimmune diseases. Two other authors, G.T.N. and P.A.C., have expressed their willingness to serve on AIRMIDs Scientific Advisory Board and to guide future clinical trials of Kv1.3 in autoimmune diseases. A 1-year option agreement with the University of California to license the University of California's Kv1.3 patents has been negotiated, although AIRMID still does not have any intellectual property related to ShK(L5)or PAP1. In addition, an investor group is in serious negotiations over the possibility that AIRMID may receive funding in the foreseeable future. The investor group has seen the data presented in this article under a confidentiality agreement, and they have conducted their due diligence regarding the Kv1.3-based technology.

This article is a PNAS direct submission.

Abbreviations: TCL, T cell line; RA, rheumatoid arthritis; APC, antigen-presenting cell; 4-AP, 4-aminopyridine; EAD, experimental autoimmune diabetes; IS, immunological synapse; PIA, pristane-induced MHC class II-restricted chronic arthritis model; INS, insulin; OA, osteoarthritis; SF, synovial fluid; PB, peripheral blood; T1DM, type-1 diabetes mellitus; MS, multiple sclerosis; MBP, myelin basic protein.

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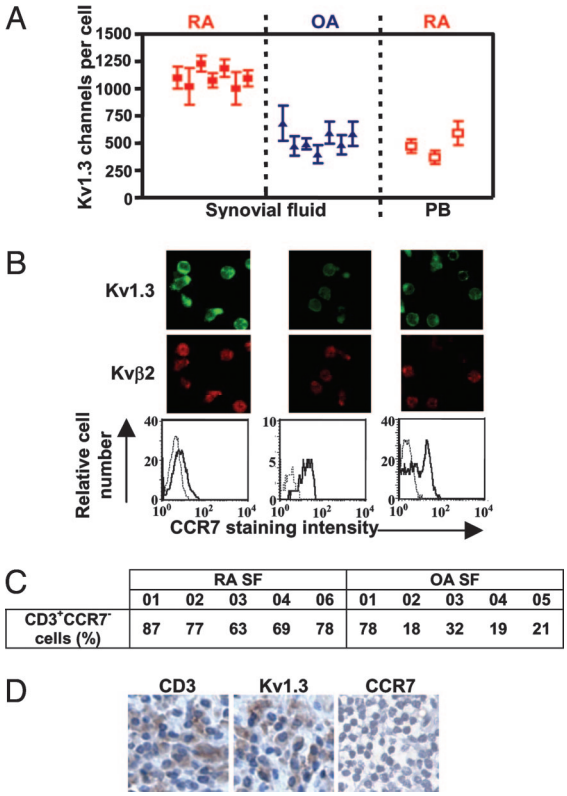

Fig. 1. Kv1.3 channel expression in RA and OA T cells. (*A*) Kv1.3 number per cell in RA-SF-T cells, RA-PB-T cells, and OA-SF-T cells. Most RA-SF-T cells were $CD4^+$ cells (Fig. 5*B*). (*B*) Confocal images of Kv1.3 (green) and Kvβ2 (red) staining in RA and OA T cells. (*C*) CCR7 expression in RA and OA T cells by FACS. (*D*) Synovial tissue from RA patients stained with anti-CD3, anti-Kv1.3, or anti-CCR7 Abs and counterstained with hematoxylin/eosin. Data are from five patients studied.

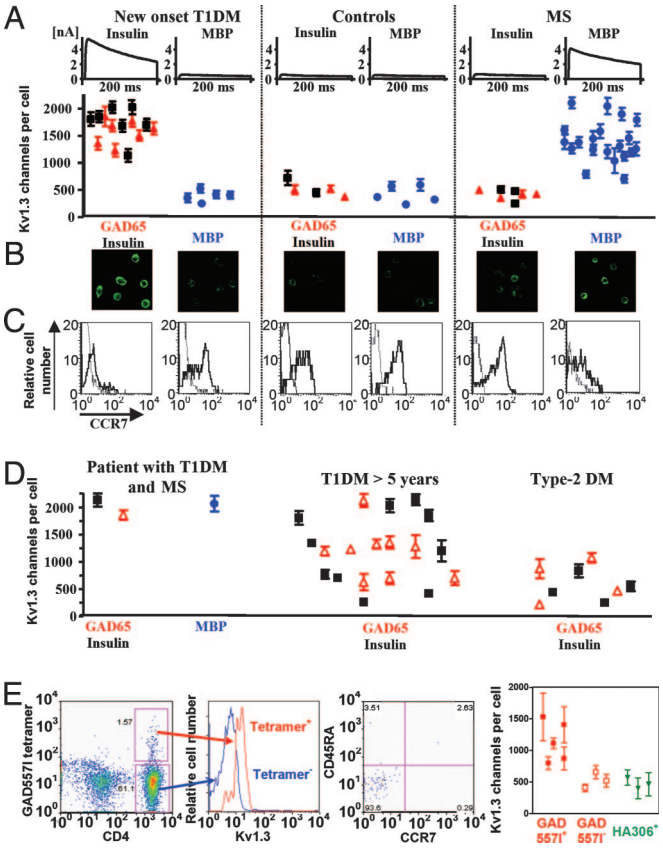

Fig. 2. Kv1.3 expression in T cells specific for GAD65 (red), INS (black), or myelin antigens (blue) from patients with T1DM, type-2 diabetes mellitus, or MS or healthy controls. (*A*) Kv1.3 currents (*Upper*) and channel number per cell (*Lower*) in activated INS-, GAD65-, and myelin-specific $CD4^+$ TCLs from new-onset T1DM patients, healthy controls, and MS patients (10). Each data point represents the mean ± SEM from 20–50 cells from two to four TCLs from a single donor. (*B*) Immunostaining for Kv1.3. (*C*) CCR7 expression in TCLs by FACS. (*D*) Kv1.3 number per cell in TCLs from a patient with both T1DM and MS and from patients with longstanding T1DM or type-2 diabetes mellitus. (*E*) Kv1.3 channel numbers per cell and CCR7, CD45R, and Kv1.3 protein expression in $CD4^+$ T cells FACS-sorted with MHC class II tetramers loaded with GAD65 557I peptide or HA 306–318 peptide from T1DM patients.

PNAS web site). The $Kv1.3^{high}$ pattern was not detected in RA-PB T cells ($P < 0.0001$) (Fig. 1*A* and Table 2) because autoreactive T cells are infrequent in the circulation and the autoantigen-specificity of these cells is unknown, making them difficult to identify. Immunostaining for Kv1.3 and its associated Kvβ2 subunit corroborated the patch-clamp data (Fig. 1*B*). Flow cytometry (FACS) demonstrated that $Kv1.3^{high}$ RA-SF-T cells were predominantly $CCR7^-$ T_{EM} cells, whereas OA-SF-T cells and RA-PB-T cells were preponderantly $CCR7^+$ naïve/T_{CM} cells (Fig. 1*C*). Immunostaining of synovial tissues from RA patients revealed many $Kv1.3^+$ and $CD3^+$ T cells (Fig. 1*D* and Fig. 5, which is published as supporting information on the PNAS web site).

We next examined Kv1.3 currents in PB-T cells from T1DM patients or controls (Table 1). Because autoantigen-specific T cells are rare in the blood (8), we used two methods to amplify relevant populations. First, we generated short-term antigen-specific $CD4^+$ T cell lines (TCLs) specific for T1DM-associated autoantigens insulin (INS; peptide 9–23) and GAD65 (peptide 555–567) or the control MS-associated autoantigen myelin basic protein (human MBP, whole protein). TCLs were patch-clamped 48 h after the third activation with the specific autoantigen. Second, we used phycoerythrin-tagged MHC class II tetramers to FACS-sort $CD4^+$ T cells from HLA-DR-0401$^+$ T1DM patients that were specific for GAD65 (555–567; 557I variant peptide) or the control antigen HA (306–318). GAD65- and INS-specific patient T cells (TCLs and tetramer-GAD65$^+$), regardless of disease activity or duration, expressed higher numbers of Kv1.3 channels (1,385 ± 210 channels per cell, n = 518 cells) compared with T cells specific for control antigens (MBP-specific TCL, tetramer-HA$^+$, tetramer-GAD65$^-$) in these T1DM patients (457 ± 25 channels per cell, $n = 90$ cells; $P < 0.001$) as well as in other controls (GAD65-/INS-/myelin-specific-TCLs from healthy controls, GAD65-/INS-specific TCLs from MS and type-2 diabetes mellitus patients) (601 ± 29 channels per cell, $n = 708$ cells; $P < 0.001$) (Fig. 2 *A*, *C*, and *D*, and Fig. 6 and Tables 1, 3, and 4, which are published as supporting information on the PNAS web site). Among the different cohorts of T1DM patients, Kv1.3 channel numbers in GAD65-/INS-specific TCLs from recent-onset T1DM patients (1,805 ± 45 channels per cell, $n = 305$ cells) were higher ($P < 0.001$) than in patients with longstanding T1DM (1,205 ± 78, $n = 157$ cells), suggesting a disappearance of $Kv1.3^{high}$ T_{EM} cells paralleling the loss of β cell antigens as the disease progresses. In one individual with both T1DM and MS, TCLs specific for GAD65, INS, and MBP all expressed high numbers of Kv1.3 channels (Fig. 2*C*). The patch-clamp data were confirmed by immunostaining for Kv1.3 (Fig. 2 *A Bottom* and *D Left*). FACS revealed $Kv1.3^{high}$ T cells to be $CCR7^-$ T_{EM} cells (Fig. 2 *B* and *D*). For comparison we have plotted our published data on antigen-specific $CD4^+$ TCLs from MS patients (10). Here too, myelin-specific TCLs were predominantly $CCR7^-Kv1.3^{high}$ T_{EM}

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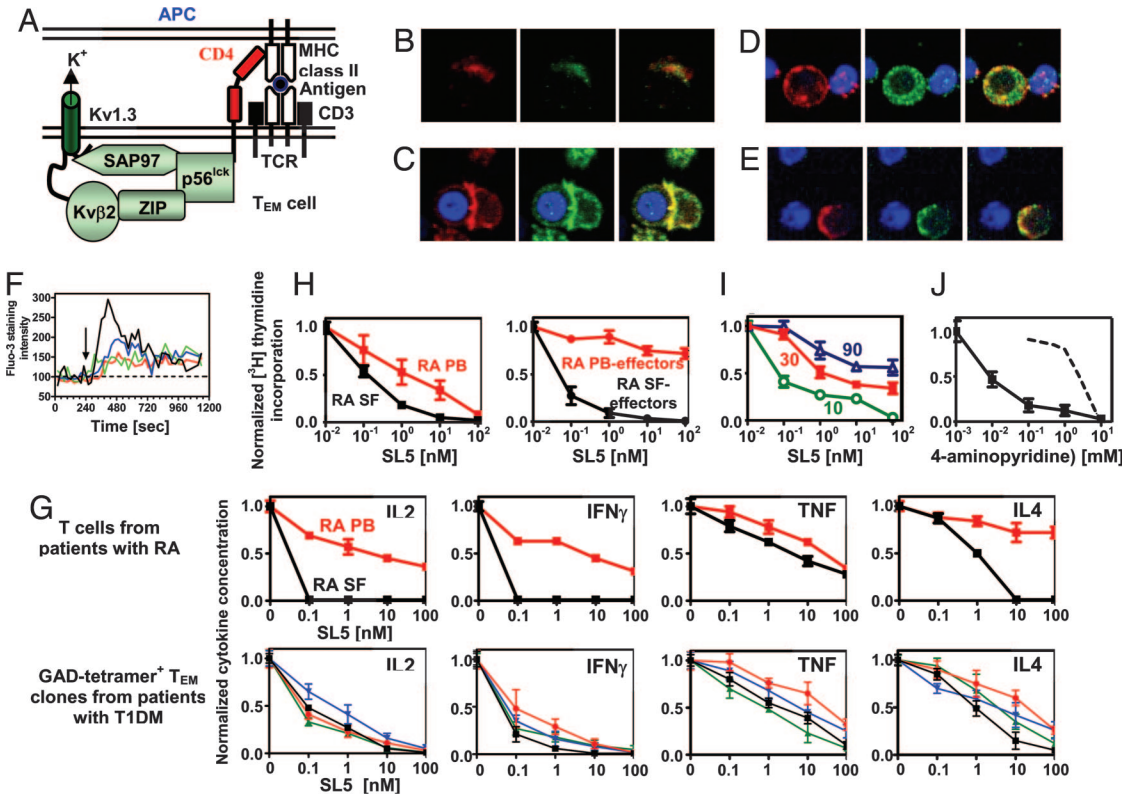

Fig. 3. Specific Kv1.3 blockers preferentially suppress human T_{EM} cells. (*A*) Kv1.3-containing signaling complex: Kv1.3, Kvβ2, SAP97 (synapse-associated protein 97), ZIP (PKC ζ-interacting protein, *p56lck*-associated p62 protein), p56lck, and CD4 (37). (*B*) Cocapping of Kv1.3 (green) with CD4 (red) in human T_{EM} cells. (*C* and *D*) CD4 (red) and Kv1.3 (green) staining in human GAD65-specific T_{EM} cells exposed to APCs loaded with GAD65 557I (*C*) or MBP (*D*). (*E*) SL5 100 nM does not prevent IS formation. (*F*) Ca^{2+} signaling in GAD-specific $CD4^+$ T_{EM} clones triggered by anti-CD3 plus cross-linking secondary Ab (arrow) in the absence (black) or presence of SL5 at 0.1 nM (blue), 1 nM (green), or 100 nM (red). (*G*) SL5 suppression of cytokine production by RA-SF-T cells, RA-PB-T cells, and tetramer-sorted GAD65-specific T_{EM} clones from T1DM patients. Amounts of cytokines produced are in Fig. 9. (*H Left*) Anti-CD3 Ab-stimulated [^{3}H]thymidine incorporation by RA-PB-T cells versus RA-SF-T cells from three RA patients. (*H Right*) [^{3}H]Thymidine incorporation by same two populations after they were stimulated for 48 h with anti-CD3 Ab, rested overnight in medium, and then rechallenged with anti-CD3-Ab. (*I*) GAD65-specific T_{EM} cells escape from Kv1.3 blockade as the amount of GAD65 557I peptide increases from 10 (green) to 30 (red) to 90 (blue) μg/ml. (*J*) Effect of 4-AP on T_{EM} proliferation induced by anti-CD3 Ab. Each point represents mean $\pm$ SD of triplicates. Dotted line shows previously published data (17) on PB-T cells from healthy donors.

cells whereas INS-/GAD65-specific TCLs were $CCR7^+Kv1.3^{low}$ naïve/T_{CM} cells (Fig. 2 *A* and *B*).

These results demonstrate that disease-associated autoreactive T cells in T1DM, MS and RA are mainly $CCR7^-Kv1.3^{high}$ T_{EM} cells, and this phenotype could serve as an important marker to distinguish autoreactive T cells between patients and control subjects (Fig. 7, which is published as supporting information on the PNAS web site). Because $CD8^+$ T_{EM}/T_{EMRA} cells also up-regulate Kv1.3 upon activation (10), we would predict that disease-related $CD8^+$ autoreactive memory T cells (14) will express the $Kv1.3^{high}$ pattern.

Specific Kv1.3 Blockers Preferentially Suppress T_{EM} Cells from RA and T1DM Patients. We used selective Kv1.3 blockers to discern whether T_{EM} cell function can be preferentially suppressed without impacting naïve/T_{CM} cells in RA and T1DM patients. Four functional parameters were measured: immunological synapse (IS) formation (22), Ca^{2+} signaling, cytokine production, and [^{3}H]thymidine incorporation. In human $CD4^+$ T_{EM} cells, Kv1.3 and its associated proteins (Fig. 3*A*) cocapped with CD4 (Fig. 3*B*), and the entire complex clustered at the IS when GAD65-specific T_{EM} clones were exposed to antigen-presenting cells (APCs) loaded with GAD65 (Fig. 3*C* and Fig. 8, which is published as supporting information on the PNAS web site), but not with the irrelevant antigen MBP (Fig. 3*D*). SL5 (20), a selective inhibitor of Kv1.3, neither prevented IS clustering (Fig. 3*E*) nor disrupted the IS once formed (Fig. 8) at a concentration that blocks >99% of Kv1.3 channels (100 nM). These data indicate that K^+ efflux through Kv1.3 channels is not necessary for IS formation or stability. IS clustering of Kv1.3 may rather provide a mechanism for channel regulation by *lck* phosphorylation (23).

SL5 inhibited Ca^{2+} signaling in GAD65-specific $CD4^+$ T_{EM} clones in a dose-dependent fashion with an IC_{50} $\approx$200 pM (Fig. 3*F*), a pharmacologically relevant concentration (20). SL5 and PAP1 (21) inhibited IL2 and IFNγ production by RA-SF-T cells (mainly T_{EM} cells) more effectively than RA-PB T cells (mainly naïve/T_{CM} cells), but these Kv1.3 inhibitors were less effective in suppressing the production of TNF-α and IL4 (Fig. 3*G Upper* and Fig. 9, which is published as supporting information on the PNAS web site). SL5 also inhibited IL2 and IFNγ production by GAD65-specific T_{EM} clones from T1DM patients (Fig. 3*G Lower*). SL5 was 10-fold more effective in suppressing [^{3}H]thymidine incorporation by RA-SF-T cells compared with RA-PB-T cells from the same patients (Fig. 3*H Left*), but when these cell populations were activated for 48 h, rested, and restimulated, RA-SF T cells remained exquisitely sensitive (IC_{50} $\approx$ 100 pM) to SL5 whereas RA-PB T cells were resistant (Fig. 3*H Right*). This "escape" by naïve/T_{CM} cells is due to up-regulation of the KCa3.1 channel that modulates Ca^{2+} signaling in activated naïve/T_{CM} cells in place of Kv1.3 (10). A significant aspect of this finding is that Kv1.3 blockers may have an advantage over

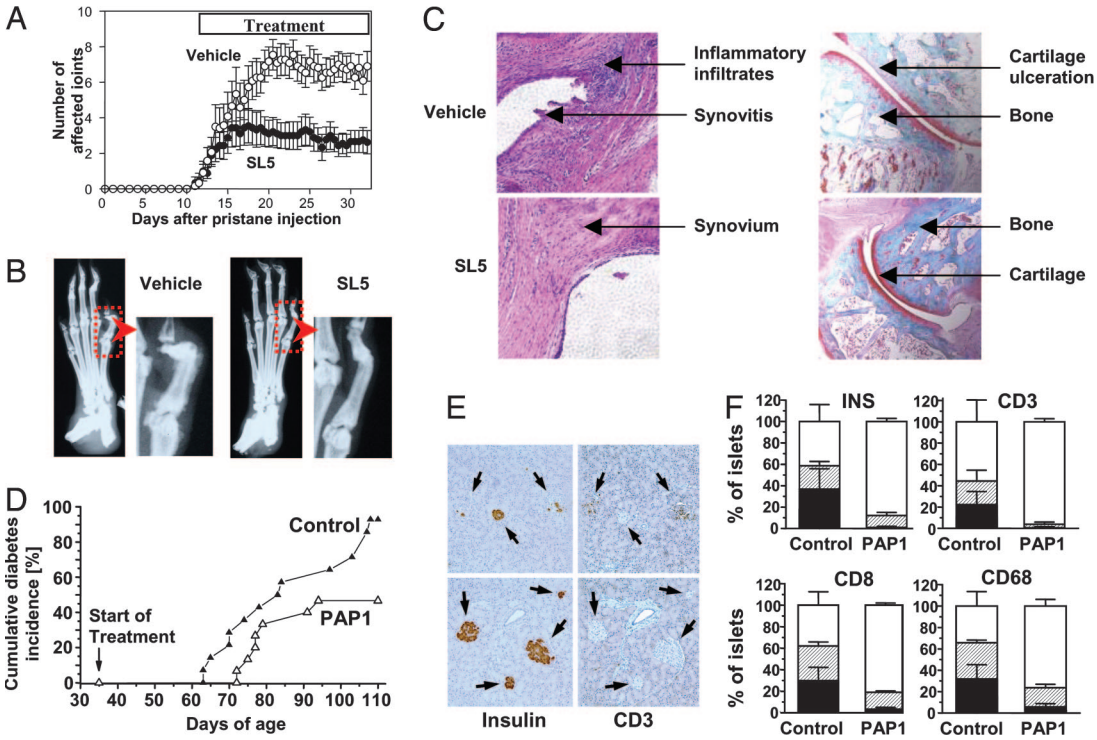

Fig. 4. Kv1.3 blockers ameliorate PIA and EAD in rats. (*A*) SL5 treatment of PIA in which vehicle-treated animals have periostitis and joint deformation. (*B*) X-rays of paws. (*C*) Staining of joints from vehicle-treated ($n = 5$) and SL5-treated ($n = 5$) rats with PIA. (*D*) Cumulative incidence of EAD. (*E*) Islets immunostained for INS (first column), CD3 (second column), CD8 (third column), and CD68 (fourth column) in vehicle-treated (*Upper*) and PAP1-treated (*Lower*) rats. (*F*) PAP1 reduces β cell destruction and intraislet infiltration by T cells and macrophages on day 70. Destruction/infiltration: none, white; moderate, gray; severe, black.

current immunomodulatory therapies because naïve and long-lived T_{CM} cells (main memory pool) would escape inhibition while T_{EM}/T_{EMRA} cells would be targeted. As confirmation of specificity for T_{EM} cells *in vivo*, SL5 (10 μg/kg per day) did not prevent the generation of skin-homing $CD3^+CD45RC^-CCR7^-$ T_{EM} cells or their migration to the site of a delayed-type hypersensitivity reaction in Lewis rats, but effectively suppressed T_{EM} function without affecting antigen-specific IgG and IgM responses (Fig. 10, which is published as supporting information on the PNAS web site).

Because all $CCR7^-$ T_{EM}/T_{EMRA} cells, regardless of antigen-specificity, up-regulate Kv1.3 channels when activated, Kv1.3 blockers might globally suppress T_{EM}/T_{EMRA} cells and compromise the ability to respond to pathogens. However, we found that GAD-specific $CD4^+CCR7^-$ T_{EM} cells from T1DM patients escaped SL5 suppression when the strength of antigenic stimulus increased (Fig. 3*I*). This result suggests that T_{EM} cells specific for pathogens and vaccine antigens are likely to overcome Kv1.3 blockade when challenged by a high amount of antigen.

Kv1.3 Inhibitors Ameliorate Disease in Rat Models of RA and T1DM. We evaluated the therapeutic potential of Kv1.3 inhibitors in rat models of RA and T1DM. Mouse models of autoimmune disease (e.g., NOD mice) are not suitable for evaluating the effects of Kv1.3 blockers because the membrane potential of mouse T cells is not regulated by K_V channels (24, 25), and the K^+ channel expression pattern of mouse T cells, particularly repeatedly activated cells, is different from that of human T cells (26, 27). SL5 and PAP1 were used in the arthritis study and the experimental autoimmune diabetes (EAD) study as representative peptide and small-molecule Kv1.3 inhibitors, respectively.

We performed a treatment trial with SL5 in the pristane-induced MHC class II-restricted chronic arthritis model (PIA) in Dark Agouti rats (28, 29). Rats were given single daily s.c. injections of vehicle (saline) or SL5 (100 μg/kg per day) at the first sign of arthritis, and therapy was continued for 21 days. The duration of the trial and the severity of arthritis were consistent with published studies (28). Rats developed arthritis around day 10, and in vehicle-treated animals ($n = 14$) disease severity worsened continuously with time (Fig. 4*A*). Radiological analysis revealed significant periostitis, erosion, and deformity, and histopathological studies showed severe synovitis, inflammatory cell infiltrate, and cartilage ulceration (Fig. 4 *A–C*). SL5-treated rats ($n = 11$) had significantly fewer affected joints during the entire course of treatment ($P < 0.05$ on days 19–34) (Fig. 4*A*) and showed significant improvement in radiological and histopathological findings (Fig. 4 *B* and *C*). Circulating levels of rheumatoid factor (IgG) and cartilage oligomeric matrix protein were not significantly reduced in treated animals (Fig. 11, which is published as supporting information on the PNAS web site). Low-titer anti-SL5 Abs were induced in these rats (Fig. 12, which is published as supporting information on the PNAS web site), but it remains to be determined whether these Abs are neutralizing and have the potential to reduce the long-term therapeutic effectiveness of SL5. No clinical signs of toxicity were identified during the trial. Kv1.3 inhibitors, like TNF-α receptor antagonists, which are the mainstay of RA therapy (30), may have to be repeatedly administered to prevent disease progression, and treatment may have to be initiated early in the disease. Because Kv1.3 blockers do not effectively suppress TNF-α production (Fig. 3*G*), a combination of a Kv1.3 blocker and a TNF-α receptor antagonist may be more effective than either therapy alone.

We conducted a prevention trial of PAP1 in MHC class II-restricted DP-BB/W rats, a standard model for T1DM. We were unable to perform a treatment study because DP-BB/W rats progress to severe ketoacidosis and death with almost complete destruction of pancreatic β cells within 1–2 days after

the onset of hyperglycemia (31, 32) if INS is not administered. In contrast, NOD diabetic mice survive for weeks without exogenous INS, and ketoacidosis is mild. DP-BB/W rats daily received vehicle (peanut oil, 3 μl/g, $n = 14$) or PAP1 ($n = 15$) at 50 mg/kg by gavage starting from 35 days of age, and treatment was continued until day 110. The duration of our trial is in agreement with published reports (33, 34). Vehicle-treated rats began developing EAD at 70 days of age with 13 of 14 animals (93%) developing EAD by day 110 (Fig. 4*D*). In contrast, only 7 of 15 rats treated with PAP1 (47%), which produced pharmacologically relevant concentrations in the blood and pancreas (Fig. 13, which is published as supporting information on the PNAS web site), developed EAD by day 110 ($P = 0.02$) (Fig. 4*D*). In comparison, anti-CD4 Ab administered from day 7 of age to DP-BB/W rats only reduced the cumulative EAD incidence from 61% to 34% (35). In a separate group of rats we evaluated the ability of PAP1 to prevent lymphocytic insulitis that destroys pancreatic β cells and precedes the development of EAD. PAP1 was administered daily from 35 to 70 days of age. In PAP1-treated rats we observed decreased intraislet T cell and macrophage infiltration and reduced β cell destruction compared with vehicle-treated controls (Fig. 4*E* and Fig. 14, which is published as supporting information on the PNAS web site). Because Kv1.3 inhibitors are reported to increase glucose uptake by mouse adipocytes by stimulating GLUT4 translocation (36), the EAD-preventing effects of PAP1 may be via increasing peripheral INS sensitivity or via effects on the production of the INS-sensitizing adipocyte hormone adiponectin. However, neither basal nor INS-stimulated glucose uptake or adiponectin secretion by isolated cultured rat adipocytes was increased by PAP1, SL5, or margatoxin (Fig. 15, which is published as supporting information on the PNAS web site), indicating that PAP1 prevents EAD in DP-BB/W rats via immunomodulation. These encouraging results coupled with results from *ex vivo* studies on disease-associated autoreactive T cells from T1DM patients (Fig. 2) provide a rationale for evaluating Kv1.3 inhibitors as a therapy for T1DM and for preventing autoimmune destruction of HLA-matched grafted islets in T1DM patients.

Safety Profile of Kv1.3 Inhibitors. A key issue for any long-term therapy is the balance between efficacy and safety. Although suppression of Kv1.3 would appear to provide a good approach to modulate pathologic immune responses mediated by autoreactive T_{EM} cells, Kv1.3 is also present in the central nervous system, kidney, liver, skeletal muscle, platelets, macrophages, testis, and osteoclasts, raising the possibility that Kv1.3 blockers could have adverse side effects. To investigate this possibility, we performed 28-day toxicity studies in rats with PAP1 (50 mg/kg), repeated doses being administered by gavage, and with SL5 repeatedly administered (100 μg/kg per day or 500 μg/kg per day) by daily s.c. injections. Both blockers failed to induce any histopathological changes in any tissue examined, including those reported to express Kv1.3 (Table 5, which is published as supporting information on the PNAS web site). However, SL5 produced skin irritation at the injection site (Table 6, which is published as supporting information on the PNAS web site). The blockers did not perceptibly alter blood cell counts or serum chemistry parameters (Tables 5 and 6). PAP1 also failed to cause signs of toxicity in treated DP-BB/W rats during 10 weeks of therapy. Rhesus macaques administered single doses of Kv1.3 inhibitors (PAP1, 3 mg/kg i.v.; SL5, 100 μg/kg i.v.) did not exhibit toxicity (A. A. Ansari, personal communication). We previously reported that SL5 and PAP1 exhibit no perceptible *in vitro* toxicity and were negative in the Ames test (20, 21), and SL5 had no effect on cardiac parameters as measured by continuous EKG monitoring (20). The relative safety of Kv1.3 blockers may be due in part to channel redundancy and also because Kv1.3 blockers may not inhibit Kv1.3-containing heteromultimers (e.g., in the CNS) with the same affinity as Kv1.3 homotetramers in T cells. More extensive toxicity studies are necessary to confirm the safety profile of Kv1.3 inhibitors. The dose limitations of combination therapy may diminish side effects without undermining the effective mechanism of individual therapies.

Several lines of evidence suggest that Kv1.3 inhibitors may not increase susceptibility to infections, although this will have to be thoroughly investigated in future. First, quinine, an antimalarial agent, blocks Kv1.3 ($IC_{50} = 14$ μM) (37) at concentrations found in patients' circulation (8–50 μM) (38). Patients that have received quinine have not exhibited an enhanced risk of infections or evidence of generalized immunosuppression (39). (Quinine blocks other channels, and its toxicity profile is consequently different from that of specific Kv1.3 inhibitors.) Second, Alefacept, an immunotherapeutic that targets T_{EM} cells (15) like Kv1.3 inhibitors, does not increase the risk of infection in treated psoriasis patients (4), and Alefacept-treated patients generate normal $CD4^+$-dependent Ab responses (e.g., increases in antitetanus toxoid titer after immunization) (40). These results suggest that suppression of T_{EM} cells by Kv1.3 inhibitors should not increase susceptibility to infection and not compromise immune responses to vaccination. Third, 4-aminopyridine (4-AP), a K^+ channel blocker used in MS therapy to augment nerve conduction, suppressed T_{EM} proliferation at concentrations ($IC_{50} \approx 8$ μM) (Fig. 3*J*) comparable to those found in treated MS patients (cerebrospinal fluid 5 μM), suggesting that the therapeutic effect of 4-AP may be mediated in part by T_{EM} suppression. 4-AP is not reported to augment susceptibility to infections or broadly immunosuppress treated MS patients. Finally, rats housed under standard (non-specific pathogen-free) conditions and repeatedly administered PAP1 or SL5 for 28 days did not develop any apparent opportunistic infections.

Advantages of Kv1.3 Inhibitors. Kv1.3 blockers preferentially suppress autoreactive $CCR7^-$ T_{EM} cells that arise as a consequence of repeated autoantigen stimulation during the development of disease, and the Kv1.3 channel, therefore, shows more specificity for autoreactive T cells than any molecular target expressed on all T cells. Kv1.3 blockers would have use in any autoimmune disease in which T_{EM} cells have been implicated. If a correlation is found between the levels of $Kv1.3^{high}$ T_{EM} cells and disease severity, it may be feasible to use sequential short-term therapy with Kv1.3 inhibitors when the numbers of $Kv1.3^{high}$ T_{EM} cells are high. Small-molecule Kv1.3-specific inhibitors would have several advantages over other immunotherapeutics including being less expensive to produce and easier to ship and store, and their oral bioavailability and relatively short half-lives would allow more rapid termination of therapy if adverse effects are observed.

Materials and Methods

Patients and T Cells. Patients' details are provided in Table 1. Methods for generating TCLs and tetramer-sorted T cells are provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

Electrophysiology. Whole-cell recordings were performed as described (10) (see *Supporting Text*).

IS Formation. APCs were loaded with GAD65 557I or MBP, and with DAPI (Molecular Probes, Eugene, OR). HLA-matched GAD-specific T_{EM} cell clones (41) were incubated in the absence or the presence of 100 nM SL5 (20) for 1 h, mixed with the antigen- and DAPI-loaded APCs, plated onto polylysine-coated glass coverslips, fixed, and stained for confocal microscopy (see *Supporting Text*).

Measurement of Ca^{2+} Influx. GAD-specific T_{EM} clones (41, 42) were loaded with 10 μM Fluo-3 AM (Molecular Probes), washed, and preincubated in the absence or the presence of increasing amounts of SL5 for 30 min. Ca^{2+} influx was induced with anti-CD3 Ab (Biomeda, Foster City, CA) followed by goat anti-mouse IgG (BD Pharmingen, San Diego, CA). Fluo-3 staining intensity was measured by FACS. At the end of each experiment ionomycin (5 μM) was added as a positive control, followed by EGTA (5 mM) as a negative control.

Toxicity Studies. DA rats received once-daily s.c. injections of SL5 (100 or 500 μg/kg per day) or saline for 28 days, followed by analysis of blood samples and histopathology assessment of formalin-fixed organs. Similar studies were performed on PAP1-treated Lewis rats.

Evaluating Kv1.3 Blockers in Rat Models of Disease. All experiments were approved by the Institutional Animal Care and Use Committee at the University of California (Irvine and Davis). Female DA rats 9–11 weeks old (Harlan-Sprague–Dawley, Indianapolis, IN) were administered 0.3 ml of pristane (Sigma, St. Louis, MO) by s.c. injection at the base of the tail. Rats were killed at the end of treatment, and their joints were x-rayed or processed for histopathology.

Female DP-BB/W rats (BRM, Worcester, MA) were considered diabetic if they had a blood glucose of >200 mg/dl on two consecutive days or a single blood glucose reading >350 mg/dl accompanied by the presence of ketone bodies. Diagnosis of EAD was confirmed for each rat at postmortem by checking for the presence of intraislet lymphocyte infiltration and β cell destruction. All nondiabetic animals were killed on day 110.

Statistical Analysis. *S*tatistical analysis was carried out with the Mann–Whitney *U* test or by one-way ANOVA.

Additional Details. Detailed descriptions of flow cytometry, cytokine production, and [^{3}H]thymidine incorporation can be found in *Supporting Text*.

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