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Kv1.3 Deletion Biases T Cells toward an Immunoregulatory Phenotype and Renders Mice Resistant to Autoimmune Encephalomyelitis

Anne R. Gocke,* Lori A. Lebson,* Inna V. Grishkan,* Lina Hu,* Hai M. Nguyen,† Katharine A. Whartenby,* K. George Chandy,‡ and Peter A. Calabresi*

Increasing evidence suggests ion channels have critical functions in the differentiation and plasticity of T cells. Kv1.3, a voltage-gated K⁺ channel, is a functional marker and a pharmacological target for activated effector memory T cells. Selective Kv1.3 blockers have been shown to inhibit proliferation and cytokine production by human and rat effector memory T cells. We used Kv1.3 knockout (KO) mice to investigate the mechanism by which Kv1.3 blockade affects CD4⁺ T cell differentiation during an inflammatory immune-mediated disease. Kv1.3 KO animals displayed significantly lower incidence and severity of myelin oligodendrocyte glycoprotein (MOG) peptide-induced experimental autoimmune encephalomyelitis. Kv1.3 was the only KV channel expressed in MOG 35–55-specific CD4⁺ T cell blasts, and no Kᵥ current was present in MOG-specific CD4⁺ T cell-blasts from Kv1.3 KO mice. Fewer CD4⁺ T cells migrated to the CNS in Kv1.3 KO mice following disease induction, and Ag-specific proliferation of CD4⁺ T cells from these mice was impaired with a corresponding cell-cycle delay. Kv1.3 was required for optimal expression of IFN-γ and IL-17, whereas its absence led to increased IL-10 production. Dendritic cells from Kv1.3 KO mice fully activated wild-type CD4⁺ T cells, indicating a T cell-intrinsic defect in Kv1.3 KO mice. The loss of Kv1.3 led to a suppressive phenotype, which may contribute to the mechanism by which deletion of Kv1.3 produces an immunotherapeutic effect. Skewing of CD4⁺ T cell differentiation toward Ag-specific regulatory T cells by pharmacological blockade or genetic suppression of Kv1.3 might be beneficial for therapy of immune-mediated diseases such as multiple sclerosis. The Journal of Immunology, 2012, 188: S877–S886.

Calcium signaling plays a critical role in T cell activation. TCR ligation is followed by a transient release of calcium from intracellular stores, which precedes a sustained calcium influx through Ca²⁺ release-activated Ca²⁺ channels that is required for complete activation. Potassium channels play a fundamental role in modulating calcium signaling by providing the counterbalancing cation flux to promote calcium entry through Ca²⁺ release-activated Ca²⁺ channels (1–3). Human T lymphocytes express two types of K⁺ channels, a voltage-gated channel composed of four Kv1.3 subunits and a calcium-activated channel composed of four KCa3.1 subunits (1). Quiescent human CD4⁺ and CD8⁺ T cells express ∼250 Kv1.3 channels and 10–20 KCa3.1 channels per T cell. Human naive and central memory T (TCM) cells upregulate KCa3.1 upon activation and are inhibited by KCa3.1-specific blockers, whereas effector memory T (TEM) cells upregulate Kv1.3 and are inhibited by Kv1.3-specific blockers (1, 4, 5). Thus, the sensitivity of lymphocyte subsets to selective blockers of Kv1.3 and KCa3.1 depends on which channel is predominantly expressed. Genetic suppression of Kv1.3 channels results in reversal of TEM to TCM cells through SMAD3/p21c1p1 signaling (6, 7). Kv1.3 clearly has an important functional role in human TEM cells.

During the course of immune, or autoimmune, stimulation, T cells receive a series of activation signals, leading to the conversion into TCM and/or TEM cells. Whereas TCM are important for the maintenance of beneficial immunity, pathogenic T cells in tissue sites of inflammation are typically TEM. Pathogenic T cells in patients with multiple sclerosis (MS), new-onset type 1 diabetes mellitus, rheumatoid arthritis, psoriasis, and acute coronary syndrome are activated TEM cells that express >1000 Kv1.3 channels/cell (4, 8–11). Importantly, pharmacological blockade of Kv1.3 channels has been shown to result in specific inhibition of clonally expanded TEM cell-mediated autoimmune responses while leaving naive and TCM immunity unchanged (4, 8, 12–15). Furthermore, Kv1.3 blockers are effective in rodent models of MS (12–15), rheumatoid arthritis (8, 16), contact dermatitis (16), type 1 diabetes mellitus (8), autoimmune glucocerebrosphatis (17), and psoriasis (10). Therefore, determining the mechanism by which this ion channel mediates its effects will not only provide insight into the novel role of ion channels in T cell lineage fate determination, but also help to elucidate the mode of action of pharmacologic blockers that may be clinically relevant. Although several selective Kv1.3 channel blockers exist, absolute specificity of a pharmacological agent is difficult to obtain, and maintaining efficient blockade for sustained periods of time while avoiding toxic concentrations of drugs is challenging. We therefore sought to use gene-specific Kv1.3 deletion in mice to assess its role on the...
of the immune system (22). Kv1.3 KO CD4+CD8+ thymocytes mouse indicated no significant role for Kv1.3 in the development study describing the generation of the Kv1.3 knockout (KO) appears to be exclusively Kv1.3 channels (1, 20, 21). An original in a 6–10-fold increase in the amplitude of the KV current, which Kv3.1 channels/cell (18–21). Activation of these subsets results important role in Ag-specific activation of CD4+ T cells during the of the inflammatory setting in the absence of this important and further characterized the phenotype of T cells following activation in an inflammatory setting in the absence of this important potassium channel. Our data demonstrate that Kv1.3 plays an important role in Ag-specific activation of CD4+ T cells during the course of EAE and show that this is due, at least in part, to intrinsic defects in the T cells themselves. Moreover, we provide the first evidence, to our knowledge, that deletion of Kv1.3 can affect the differentiation of CD4+ T cells, resulting in the development of CD4+ T cells with regulatory properties. These results indicate that specific and effective blockade of Kv1.3 could be beneficial for the treatment of inflammatory immune-mediated diseases such as MS.

Materials and Methods

Mice

Female C57BL/6, D2D TCR-transgenic, and CD45.1 congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a federally approved animal facility at The Johns Hopkins University (Baltimore, MD) in accordance with the Institutional Animal Care and Use Committee. Kv1.3 KO mice on the C57BL/6 background were a kind gift from Dr. Leonard Kaczmarek (Yale University, New Haven, CT) and were bred and maintained in our animal facility. All mice were between 7 and 10 wk of age when experiments were performed. Age- and sex-matched C57BL/6 mice or littermates were used in all experiments as controls.

EAE induction

EAE was induced in WT and Kv1.3 KO mice by s.c. injection over two sites in the flank with 100 μg myelin oligodendrocyte glycoprotein (MOG 35–55) peptide (The Johns Hopkins Peptide Synthesis Core Facility) emulsified in CFA (Difco, Detroit, MI). A total of 250 μg pertussis toxin (List Biologicals, Campbell, CA) administered at the time of immunization and 48 h later by i.p. injection. Mice were monitored daily for clinical signs of EAE and given a clinical score based on the following criteria: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, moribund; and 5, death due to EAE. To measure T cell expansion in vivo, CD45.2 CD4+ T cells from Kv1.3 KO and WT mice were isolated and injected i.v. into WT CD45.1 recipients. The recipients were then immunized to induce EAE as described above.

CD4+ T cell isolation

Spleens and lymph nodes were isolated from Kv1.3 KO or WT mice, and single-cell suspensions were made by passing through a 70-μm nylon cell strainer (BD Biosciences, San Jose, CA). CD4+ T cells were then isolated by negative selection using an EasySep CD4+ T cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. Briefly, a biotinylated mouse CD4+ T lymphocyte enrichment mixture was added to the cell suspension. Addition of this mixture results in labeling of leukocytes that are not CD4+ T cells. Magnetic streptavidin particles were then added to the suspension, and all labeled cells migrated toward a magnet, leaving the unlabeled CD4+ T cells in suspension. The CD4+ T cells were retained and all other cells discarded. Following isolation, cells were washed, counted, and resuspended in complete RPMI 1640 media for downstream applications.

Dendritic cell culture

Bone marrow-derived dendritic cells (DCs) were generated by standard methods as follows: bones were flushed with RPMI 1640/10% FCS (both from Invitrogen, Carlsbad, CA), and a single-cell suspension was prepared. Following centrifugation, cells were resuspended in DC medium (RPMI 1640 containing 10% FBS, sodium pyruvate [Sigma-Aldrich, St. Louis, MO], penicillin/streptomycin [Quality Biological, Gaithersburg, MD], and 1% HEPES buffer [Invitrogen], with 20 ng/ml GM-CSF [Peprotech, Rocky Hill, NJ]). Cells were plated in non–tissue-culture Petri dishes (100 mm) at 2.5 × 10^6/plate. On day 7, cells were stimulated in the presence or absence of LPS (5 ng/ml) overnight. On day 8, DCs were collected for analysis.

Tritiated thymidine incorporation proliferation assay

Draining lymph nodes from Kv1.3 KO or WT mice were harvested 7 d postimmunization, and single-cell suspensions were made by passing through a 70-μm cell strainer (BD Biosciences). CD4+ T cells were isolated as described above, and 1 × 10^6 cells/well were plated in a 96-well flat-bottom plate. T cells were restimulated with increasing concentrations of MOG 35–55 and 3 × 10^5 irradiated APCs/well. To assess antigen presentation capabilities of Kv1.3 KO DCs or DCs from Kv1.3 KO or WT mice were generated from the bone marrow of mice using standard protocol of culturing with GM-CSF, as described above. These DCs were then pulsed with 5 μg/ml MOG 35–55 peptide. MOG-pulsed DCs were cultured with CD4+ T cells from 2D2 mice in increasing ratios. Cultures were maintained for 96 h at 37°C in humidified 5% CO2/air. The cells were pulsed with 0.5 μCi/well [methyl-^3H]thymidine for the final 16 h of culture. Cells were harvested on glass filters, and incorporated [methyl-^3H] thymidine was measured with a Betaplate counter (PerkinElmer Wallac, Gaithersburg, MD). Background levels of proliferation from cells that were not stimulated with Ag were subtracted to determine the proliferation for each condition. The results were determined as means from quadruplicate cultures and are shown with SEM.

CFSE proliferation assay

Following isolation, Kv1.3 KO or WT CD4+ T cells were labeled with CFSE (Molecular Probes, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, cells were resuspended at a concentration of 1 × 10^7/ml in 0.1% BSA in PBS and incubated with 0.25 μM CFSE at 37°C for 10 min. The staining was quenched with addition of five volumes of culture medium. Cells were cultured with CD4+ T cells from 2D2 mice in increasing ratios. Cultures were maintained for 96 h at 37°C in humidified 5% CO2/air. The cells were pulsed with 0.5 μCi/well [methyl-^3H]thymidine for the final 16 h of culture. Cells were harvested on glass filters, and incorporated [methyl-^3H] thymidine was measured with a Betaplate counter (PerkinElmer Wallac, Gaithersburg, MD). Background levels of proliferation from cells that were not stimulated with Ag were subtracted to determine the proliferation for each condition. The results were determined as means from quadruplicate cultures and are shown with SEM.

Cell-cycle analysis

A BrdU flow kit (BD Pharmingen, San Diego, CA) was used to determine the cell-cycle kinetics. The assay was performed according to the manufacturer’s protocol. Briefly, cells were stimulated with anti-CD3/CD28 beads (1 × 10^6/well), and 10 μM BrdU was added for the final 18 h of culture. Cells were fixed in a solution containing paraformaldehyde and the detergent saponin and incubated for 30 min. The staining was quenched with addition of five volumes of culture media containing 10% FBS. The cells were centrifuged, washed, and resuspended in complete RPMI 1640 media. The cells were then plated at 1 × 10^7/ml and stimulated with 5 μg/ml Dynal anti-CD3/CD28 beads (Invitrogen). The cells were cultured for 72 h, stained with an anti-CD4–APC Ab (BD Biosciences), and run on the FACS caliber flow cytometer (BD Biosciences) to assess cell division. The data were analyzed using FlowJo software (Tree Star, Ashland, OR).
Electrophysiology

CD4+ T cells from MOG-immunized WT and Kv1.3 KO mice were collected 8 d postimmunization and restimulated ex vivo with MOG 35–55 peptide. Cells were then cryopreserved and shipped to the University of California, Irvine, for patch clamping. Cells were thawed and patch-clamp configuration was maintained by a Ca2+-free pipette solution containing (in mM): 145 KF, 10 HEPES, 10 EGTA, and 2 MgCl2 (pH 7.2, 300 mOsm). Kv1.3 currents were elicited by repeated 200 ms pulses from a holding potential of −80–40 mV, applied every second to visualize Kv1.3’s characteristic cumulative inactivation (4, 23) or every 30 s to avoid inactivation of Kv1.3 channels, in experiments measuring blocking by a Kv1.3-specific inhibitor, ShK-186, at 100 pM and 1 nM (15). Whole-cell Kv1.3 conductances were calculated from the peak current amplitudes at 40 mV. Kv1.3 channel numbers per cell were determined by dividing the whole-cell Kv1.3 conductance by the single-channel conductance value for each channel (Kv1.3, 12 pS) (4, 23, 24). Cell capacitance, a direct measure of cell surface area, was constantly monitored during recording. Statistical differences in channel number per cell were determined by Student t test.

Perfusion and immunostaining

Kv1.3 KO or WT EAE mice were anesthetized, perfused with HBSS, and spleens and brains were removed. CNS tissue was digested and enriched for mononuclear cells. Single-cell suspensions were made from spleens as described above. Collected cells were stimulated in media containing PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich), and monensin (4 μg/ml; GolgiStop; BD Biosciences) at 37°C for 4–6 h. Cells were washed with FACS buffer (2% FBS in PBS) and surface stained with anti-CD3 PerCP or APC, anti-CD4 PerCP or APC, anti-CD44 FITC, anti-CD62L PE, anti-CD25 FITC, anti-CD45.2 FITC, Vβ11 PE, and Annexin V (BD Biosciences and eBioscience, San Diego, CA). Cells were then fixed and permeabilized using the Foxp3 staining kit (eBio-PE, and Annexin V (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).}

ELISA

CD4+ T cells were isolated from unimmunized Kv1.3 KO or WT mice as described above and stimulated for 72 h or 4 d followed by a 3-d rest period with Dynal anti-CD3/CD28–coated beads (Invitrogen) for a total of three cycles. Supernatants were collected 72 h following the first and third stimulation, and cytokine secretion was measured by ELISAs. DCs generated from bone marrow of WT or Kv1.3 KO mice were stimulated with or without 5 ng/ml LPS overnight, and IL-6 production was measured by ELISA. IFN-γ, IL-17, IL-10, and IL-6 ELISAs were performed using mouse ELISA kits specific for the aforementioned cytokines according to the manufacturer’s protocols (BioLegend, San Diego, CA, and R&D Systems, Minneapolis, MN).

Western blotting

CD4+ T cells were isolated from unimmunized Kv1.3 KO or WT mice and stimulated with anti-CD3/CD28–coated beads for 4 d followed by a 3-d rest period. Cells were restimulated and rested in the same manner for a total of three cycles, followed by a final 6-h stimulation. Cells were collected and lysed with RIPA buffer (Boston Bioproducts, Ashland, MA) to generate whole-cell lysate. Protein was quantitated using the BCA assay (Pierce, Rockford, IL). Lysates were electrophoretically separated using 12% Tris HC1 gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Western blotting was performed using the following Abs: p-Smad3, Smad3, Actin, and goat anti-rabbit IgG-HRP (Cell Signaling Technologies, Danvers, MA). Average densitometric ratio was calculated for each blot by normalizing to an actin loading control using ImageJ software (National Institutes of Health).

In vitro suppression assay

CD4+ T cells from unimmunized Kv1.3 KO or WT mice were stimulated with anti-CD3/CD28 beads for three rounds as described above. Immediately preceding the third stimulation, Kv1.3 KO or WT T cells were incubated with WT naive CD4+ CFSE-labeled responder cells at a ratio of suppressors/responders of 5:1. The cells were stimulated with anti-CD3/CD28–coated beads for an additional 72 h and proliferation of responder cells was assessed using an FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). For ex vivo suppression assays, CD4+ T cells were isolated from MOG 35–55-immunized Kv1.3 KO or WT mice 22 d postimmunization and incubated with WT naive CD4+ CFSE-labeled responder cells at a ratio of suppressors/responders of 5:1. The cells were stimulated with anti-CD3/CD28–coated beads for an additional 72 h and proliferation of responder cells was assessed using an FACSCalibur (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (Tree Star).

Statistics

Statistical evaluation of significance between the experimental groups was determined by Student unpaired t test, Mann–Whitney U nonparametric analysis, or ANOVA as appropriate using GraphPad Prism (GraphPad, San Diego, CA). Results were determined to be statistically significant when p < 0.05: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Kv1.3 KO mice have a decreased incidence and severity of EAE and a limited proliferative capacity of lymphocytes

To investigate the effect of specific inhibition of Kv1.3 on CD4+ T cell differentiation during an immune-mediated disease, we induced EAE in Kv1.3 KO and WT C57BL/6 mice by immunization with MOG 35–55 peptide. Interestingly, WT mice developed severe EAE accompanied by loss of weight, whereas Kv1.3 KO mice had significantly decreased incidence and severity of EAE and no loss of weight over the course of the experiment (Fig. 1A, 1B). Moreover, Kv1.3 KO mice that did develop signs of EAE had a delayed onset of disease and recovered more completely and at a faster rate than WT controls (data not shown).

To determine whether protection from EAE was mediated by the inability of Kv1.3 KO T cells to become activated, we compared the numbers of CD4+ naive T cells (CD44hiCD62Llo), CD4+CD8− cells in the thymus and similar percentages of CD4+ and CD8+ T cells in the spleen and lymph node (Supplemental Fig. 1A, 1B). Unimmunized animals were also found to have similar numbers of naive, TCM, and TEM cells in the spleen and lymph node as determined by expression of CD62L and CD44 (Supplemental Fig. 1C). In contrast, MOG-immunized Kv1.3 KO mice had significantly more naive and TCM cells and fewer TEM cells in the lymph node at 7 d postimmunization than WT controls (Fig. 1C, 1D). In addition, Kv1.3 KO mice had significantly fewer activated CD4+ T cells, as characterized by change in expression of CD25 in Foxp3+ cells (gating strategy shown in Supplemental Fig. 2) in the lymph node 7 d following immunization (Fig. 1E). These observations were further validated by the relative lack of proliferation of CD4+ T cells from Kv1.3 KO mice following rechallenge with the immunizing Ag ex vivo, as compared with WT cells (Fig. 1F). These findings suggest that CD4+ T cells from Kv1.3 KO mice do not become fully activated following antigenic challenge in vivo and, as a result, retain a naive phenotype and have a limited proliferative capacity.

Kv1.3 KO mice are deficient in IFN-γ and IL-17 production and display decreased accumulation of encephalitogenic cells in the brain

To further characterize the phenotype of the CD4+ T cells from Kv1.3 KO mice during EAE, we performed intracellular cytokine staining at the peak of disease (14 d postimmunization) in CD4+ T cells isolated from spleens and brains of Kv1.3 KO and WT mice. Splenic CD4+ T cells from unimmunized Kv1.3 KO and WT mice did not secrete significant amounts of IFN-γ or IL-17.
Kv1.3 KO mice have a decreased incidence and severity of EAE and a limited proliferative capacity of lymphocytes. (A) Kv1.3 KO or WT C57BL/6 mice were immunized with MOG 35–55 peptide and monitored daily for disease severity. Mean clinical scores of animals are depicted in the graph. Incidence of disease is shown in the upper left corner. (B) Kv1.3 KO and WT mice were weighed daily following immunization. Weight in grams is depicted graphically and correlates with onset of EAE. (C and D) Four mice per group were sacrificed at 7 d postimmunization, and draining lymph nodes were stained directly ex vivo for CD4, CD44, and CD62L. Cells were gated on live, CD3+, and CD4+, and percent of each subtype, T effector memory (TEM CD44hiCD62Llo), T central memory (T CM CD44hiCD62Lhi), and T naive (CD44loCD62Lhi), are shown on the graph. (E) Lymph nodes were isolated from Kv1.3 KO and WT animals as described in (C) and stained for the activation marker CD25. Cells were gated on CD4+ Foxp3+, and CD25+ cells are shown as a histogram (left panel) and graphed as percent of CD4+Foxp3+ cells that are CD25+ (right panel). (F) CD4+ T cells from the draining lymph nodes of Kv1.3 KO and WT mice at 7 d postimmunization were restimulated in vitro with increasing concentrations of MOG 35–55 in the presence of irradiated APCs. Proliferation was measured by tritiated thymidine incorporation. All data shown are representative of three independent experiments. Statistical significance was assigned with *p < 0.05, **p < 0.01.

(Kv1.3 KO) However, in mice immunized to induce EAE, there was a significant decrease in the production of proinflammatory IFN-γ and IL-17 cytokines from CD4+ T cells in the spleens of Kv1.3 KO mice compared with WT controls (Fig. 2A, 2B). This decrease in IFN-γ and IL-17 correlated with the decreased incidence and severity of disease in the KO mice.

Because we wanted to determine whether the protection from EAE was associated with a defect in activation and recruitment of encephalitogenic cells to the CNS, we investigated whether the loss of Kv1.3 drove a change in cytokine secretion from CD4+ T cells in the brains of immunized mice. We found a significant decrease in the percentage of CD4+ T cells (WT: 4.7 ± 0.8%; KO: 2.2 ± 0.5%) in the brains of Kv1.3 KO mice compared with WT mice; further, of the cells that were present, significantly fewer were producing IFN-γ and IL-17 (Fig. 2C, 2D). These data suggest that CD4+ T cells from Kv1.3 KO mice are impaired in their ability to become fully activated in response to antigenic stimulation and, therefore, do not readily migrate to the CNS.

Kv1.3 is the only voltage-gated K+ current in MOG 35–55 restimulated CD4+ T cell blasts

We used whole-cell patch-clamp to characterize the Kv channel expression in disease-associated MOG 35–55-stimulated CD4+ T cell blasts from the spleens of WT mice with EAE. Eight days following immunization with MOG 35–55, mice with EAE were euthanized, CD4+ T cells were isolated from the spleen, restimulated ex vivo with MOG 35–55 peptide for 48 h, and frozen, and patch-clamp studies were performed. Robust K+ currents (Fig. 3A) with properties closely resembling Kv1.3 were detected (cell capacitance >4 pF, 11-μm diameter). They exhibited cumulative inactivation, a unique property of Kv1.3 that distinguishes it from other voltage-gated K+ channels (Fig. 3C). The half-maximal ac-
tivation voltage of the current was similar to that of cloned Kv1.3 (Fig. 3B) (24). The specific Kv1.3 inhibitor ShK-186 (4) completely inhibited the T cell current (Fig. 3D). The CD4+ MOG-restimulated WT blasts expressed 336 ± 23 Kv1.3 channels/cell (Fig. 3F). These results indicate that Kv1.3 is the only voltage-gated K+ current in MOG-activated WT CD4+ T cell blasts and confirms earlier work (20, 21). Calcium-activated KCa3.1 channels were not detected in these experiments because the pipette solution contained EGTA and no calcium.

Immunization of Kv1.3 KO mice with MOG 35–55 resulted in a significantly decreased incidence and severity of EAE (Fig. 1A). Cell-cycle progression was analyzed by measuring incorporated BrdU and total DNA levels (7-AAD) by flow cytometry (Fig. 4B, 4C). Roughly 18% of activated WT CD4+ T cells were in the G2/M phase, and 24% reached the S phase of the cell cycle (Fig. 4B, 4C). In Kv1.3 KO CD4+ T cells, significantly more cells remained in the G2/M phase (∼25%), and fewer cells reached the S phase (11%) (Fig. 4B, 4C), indicating that cell-cycle delay contributes to the diminished proliferative capacity of Kv1.3 KO CD4+ T cells. CFSE proliferation assays confirmed this finding. CD4+ T cells isolated from Kv1.3 KO mice and stimulated with anti-CD3/CD28/CD282 proliferated more slowly than CD4+ T cells from WT mice (Fig. 4D). No increase in apoptosis of Kv1.3 KO CD4+ T cells was noted in comparison with controls (Fig. 4E), suggesting that the slower proliferation rate of CD4+ T cells from Kv1.3 KO mice is not due to increased rate of cell death.

**DCs from Kv1.3 KO mice can activate WT CD4+ T cells and are not deficient in IL-6 production in vitro**

Activation of CD4+ T cells following immunization with MOG 35–55 requires Ag presentation by DCs to T cells. Because DCs express Kv1.3 channels (25, 26), deletion of Kv1.3 might result in improper Ag presentation by DCs and thereby contribute to defective activation of CD4+ T cells and reduced EAE in immunized Kv1.3 KO mice. To test this possibility, we isolated CD4+ T cells from unimmunized CD45.2 Kv1.3 KO or WT mice and injected these cells i.v. into WT CD45.1 congenic hosts. The recipients were then immunized to induce EAE, and the expansion of CD45.2 CD4+ T cells was measured at 14 d postimmunization from cells in the lymph node and spleen using flow cytometry. Although a modest expansion of CD45.2 CD4+ T cells was seen in
WT  
KO  

**FIGURE 4.** CD4+ T cells from Kv1.3 KO mice have an intrinsic defect in activation that is not associated with increased cell death. (A–C) CD4+ T cells were isolated from spleens of 10-wk-old Kv1.3 KO or WT mice and stimulated with anti-CD3/CD28 beads. Cultures were pulsed with 10 μM of BrdU for the final 18 h of stimulation. Cell-cycle progression was analyzed by examining incorporated BrdU (A, B) and total DNA levels (7-AAD) (B) by flow cytometry. Unstimulated cells and CD4+ T cells containing no BrdU were used as negative controls. The results from three independent experiments are depicted in a bar graph (C). (D) CD4+ T cells were isolated from spleens of 10-wk-old unimmunized Kv1.3 KO and WT mice. Cells were labeled with CFSE and stimulated with anti-CD3/CD28 beads for 72 h. Flow cytometry was performed to assess the amount of cell division as measured by CFSE dilution compared with unstimulated cells. CD4+ T cells from Kv1.3 KO mice divided more slowly than cells from WT mice with 57 versus 78% division, respectively. (E) CD4+ T cells were isolated from Kv1.3 KO and WT mice and stimulated as described in (A). Cells were stained for CD4 and Annexin V to measure cell death. There was no difference detected in the number of apoptotic CD4+ T cells between Kv1.3 KO and WT mice. All data are representative of three independent experiments. Statistical significance was determined with *p < 0.05.

mice receiving cells from WT mice, this population of cells was virtually absent in mice receiving CD4+ T cells from Kv1.3 KO mice (Fig. 5A). These data suggest that an intrinsic defect in CD4+ T cells rather than a defect in APCs is responsible for the inability of Kv1.3 KO mice to mount an effective MOG 35–55-specific immune response and develop EAE.

To investigate directly the effect of Kv1.3 deletion on the ability of DCs to present Ag and activate T cells, we performed an in vitro proliferation assay using DCs generated from the bone marrow of WT or Kv1.3 KO mice. WT or Kv1.3 KO DCs were pulsed with 5 μg/ml of MOG 35–55 peptide, plated with 2D2 TCR-transgenic CD4+ T cells, and the proliferation of the T cells was measured using tritiated thymidine incorporation. DCs generated from Kv1.3 KO bone marrow induced T cell proliferation to the same extent as WT DCs (Fig. 5B), suggesting that deletion of Kv1.3 in DCs does not impair their ability to present Ag and activate T cells.

IL-6 production by DCs during the course of EAE is believed to be important for disease pathogenesis (27). To determine whether decreased production of IL-6 contributed to the decreased incidence and severity of EAE that we observed in our Kv1.3 KO mice, DCs generated from Kv1.3 KO or WT mice were stimulated with LPS overnight, and IL-6 production was measured by ELISA. There was no difference in LPS-induced IL-6 production from WT and Kv1.3 KO mice (Fig. 5C), suggesting that deficiency in IL-6 production from DCs does not contribute to the decreased severity of EAE in the Kv1.3 KO animals.

**CD4+ T cells from Kv1.3 KO mice secrete more IL-10 in vitro and ex vivo following induction of EAE**

Because CD4+ T cells from Kv1.3 KO mice were characterized by a delayed or decreased proliferative capacity, we examined whether they exhibited properties common to regulatory T cells (Tregs). Tregs proliferate more slowly than other types of effector T cells and produce more IL-10 and less IL-17 and IFN-γ, which contributes to their ability to suppress autoreactive effector T cells (28). Moreover, anergic T cells have been shown to act as suppressor cells in vitro (29, 30). Therefore, it is possible that the Kv1.3 KO CD4+ T cells are anergic, and as a result, act as suppressors in vivo and in vitro. To determine whether CD4+ T cells from Kv1.3 KO mice have a regulatory phenotype, we isolated CD4+ T cells from the spleens of Kv1.3 KO and WT mice 14 d postimmunization and performed intracellular cytokine

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**FIGURE 5.** DCs from Kv1.3 KO mice can activate WT CD4+ T cells and are not deficient in IL-6 production in vitro. (A) Approximately 5 × 10^6 WT or Kv1.3 KO CD45.2+CD4+ T cells were injected i.v. into WT CD45.1 mice. Recipient mice were immunized with MOG 35–55 peptide. Mice were sacrificed at 14 d postimmunization, and the lymph nodes and spleen were removed. Cells were stained for CD4 and CD45.2, and the number of expanding CD45.2 cells was examined using flow cytometry. Representative FACS plots are shown with quantitative bar graphs. (B) DCs were generated from the bone marrow of Kv1.3 KO or WT mice and pulsed with 5 μg/ml of MOG 35–55 peptide. The DCs were used to stimulate MOG-specific CD4+ T cells from 2D2 TCR-transgenic mice in increasing ratios. Proliferation of T cells was measured using a tritiated thymidine assay. DCs alone or CD4+ T cells alone were used as negative controls. Kv1.3 KO and WT DCs induced proliferation of CD4+ 2D2 TCR-transgenic T cells to the same extent. (C) DCs generated from the bone marrow of Kv1.3 KO or WT mice were stimulated overnight with 5 ng/ml of LPS. Supernatants were collected, and ELISA was performed to measure IL-6 secretion. DCs from Kv1.3 KO and WT mice secreted equivalent amounts of IL-6 following LPS stimulation. *p < 0.05.
staining for IL-10. CD4+ T cells from spleens of unimmunized Kv1.3 KO and WT mice did not produce significant amounts of IL-10 and had an equal number of Foxp3+ cells (Supplemental Fig. 1E). In contrast, CD4+ T cells from Kv1.3 KO mice immunized to induce EAE made significantly more IL-10 than WT controls, consistent with a regulatory phenotype (Fig. 6A, 6B). Interestingly, this increase in IL-10 production was not associated with an increase in the frequency of Foxp3+ cells, suggesting that this population of cells differs from canonical induced Treg.

We confirmed enhanced IL-10 secretion by CD4+ T cells from Kv1.3 KO mice independent of effects from APCs. CD4+ T cells from unimmunized Kv1.3 KO or WT mice were subjected to one to three rounds of in vitro stimulation with anti-CD3/CD28 beads, in which each round consisted of activation for 3 to 4 d followed by a rest period of 3 d. The multiple rounds of stimulation were performed to generate T cells with an effector memory phenotype, as effector memory cells have been demonstrated to be more dependent on Kv1.3 signaling. Supernatants were collected 72 h after the first or third round of stimulation, and IL-10, IFN-γ, and IL-17 production was measured by ELISA. CD4+ T cells from Kv1.3 KO mice made significantly more IL-10 than cells from WT mice with a correlating decrease in IFN-γ and IL-17 production after one (Fig. 6C) or three (Fig. 6D) rounds of stimulation in vitro. These data suggest that CD4+ T cells from Kv1.3 KO mice have impaired effector functions and intrinsic regulatory properties.

CD4+ T cells from Kv1.3 KO mice acquire regulatory properties and suppress activation of naïve T cells in vitro and ex vivo

To further evaluate the regulatory capabilities of Kv1.3 KO T cells, we performed functional assays in vitro and directly ex vivo following induction of EAE. We previously reported that genetic silencing of Kv1.3 by a dominant-negative Kv1.1 lentivirus induces an increase in expression of phosphorylated SMAD3 in human CD4+ T cells (7). Phosphorylation of SMAD3 occurs in response to TGF-β signaling and has been shown to be important for the development of Treg (31–33). Furthermore, phosphorylation of SMAD3 and its association with SMAD4 has been proposed to induce transcription of IL-10 in murine CD4+ T cells (34). We therefore examined whether enhanced IL-10 production by Kv1.3 KO CD4+ T cells was a result of increased SMAD3 phosphorylation. Phosphorylation of SMAD3 was measured by Western blotting in CD4+ T cells from WT and Kv1.3 KO mice that had undergone three rounds of in vitro stimulation with anti-CD3/CD28 beads. CD4+ T cells from Kv1.3 KO mice had increased expression of phosphorylated SMAD3 compared with WT T cells (Fig. 7A, 7B). This finding supports the possibility that deletion of Kv1.3 skews the differentiation of CD4+ T cells toward a regulatory phenotype.

A functional characteristic that is a hallmark of a regulatory cell is its capability to suppress effector T cells. We performed a functional suppression assay to determine whether Kv1.3 KO CD4+ T cells exhibit suppressor activity. CD4+ T cells from WT and Kv1.3 KO mice were subjected to three rounds of stimulation. These suppressor cells were then incubated with CFSE-labeled WT CD4+ responder cells at a ratio of 5:1. The mixture of cells was stimulated with anti-CD3/CD28 beads for 72 h, and proliferation of responder cells measured using flow cytometry. CD4+ T cells from Kv1.3 KO mice suppressed the activation of WT CD4+ T cells, with ~23% of cells remaining undivided compared with 5% in controls (Fig. 7C). Unstimulated CD4+ T cells isolated from unimmunized Kv1.3 KO mice did not suppress the activation of WT CD4+ T cell responders, suggesting that the suppressive properties of Kv1.3 KO T cells are acquired only after stimulation (Supplemental Fig. 1F).

To confirm the ability of Kv1.3 KO CD4+ T cells to suppress effector cells in vivo, CD4+ T cells were isolated from Kv1.3 KO or WT mice 22 d postimmunization with MOG 35–55. These cells were plated with CFSE-labeled WT CD4+ T cells in the presence of anti-CD3/CD28 beads, and the proliferation of responder cells was measured after 72 h using flow cytometry. As seen in vitro, CD4+ T cells from Kv1.3 KO MOG-immunized mice suppressed effector CD4+ T cells, with ~50% of cells remaining undivided compared with 14% in controls (Fig. 7D). Interestingly, this suppressive phenotype did not correlate with an increase in the percentage of Foxp3+ cells, consistent with the idea that these suppressor cells differ from canonical Treg (Fig. 7E). These data further support the hypothesis that CD4+ T cells from Kv1.3 KO mice have the propensity to acquire a regulatory phenotype, which could account for the limited proliferative capacity of T cells and the lower incidence and severity of EAE in Kv1.3 KO animals.

Discussion

Controlled regulation of T cell activation in the context of inflammation has been the topic of numerous investigations through the years. The data presented in this study provide evidence that effective targeting of the Kv channel Kv1.3 on CD4+ T cells could

FIGURE 6. CD4+ T cells from Kv1.3 KO mice secrete more IL-10 in vitro and ex vivo following induction of EAE. (A) Spleens were isolated from Kv1.3 KO and WT mice at 14 d postimmunization and stained directly ex vivo for CD4 and IL-10. Cells were gated on live CD4+, and the percentage of CD4+ T cells secreting IL-10 was determined. Representative FACS plots are shown. (B) Percentage of CD4+ T cells secreting IL-10 in all mice tested is shown on a bar graph. Kv1.3 KO mice secreted significantly more IL-10 than WT controls. CD4+ T cells from 10-wk-old unimmunized Kv1.3 KO or WT mice were subjected to one (C) or three (D) rounds of stimulation in vitro and ex vivo following induction of EAE. (C) Percentage of CD4+ T cells secreting IL-10 was determined by ELISA. (D) Percentage of CD4+ T cells secreting IFN-γ and IL-17 was measured after 72 h using flow cytometry. As seen in vitro, CD4+ T cells from Kv1.3 KO mice made significantly more IL-10 than WT controls, consistent with a regulatory phenotype (Fig. 6A, 6B). Interestingly, this increase in IL-10 production was not associated with an increase in the frequency of Foxp3+ cells, suggesting that this population of cells differs from canonical induced Treg.

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Discussion

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result in the development of T cells with decreased proliferative capacity and a regulatory phenotype that would be beneficial for controlling unwanted immune responses.

We used Kv1.3 KO mice to determine the specific contribution of the Kv1.3 channel to T cell activation and differentiation during the course of MOG-induced EAE. We found that Kv1.3 KO mice were resistant to the development of EAE with significantly decreased incidence and severity of disease compared with WT controls. Upon further examination, we found that CD4+ T cells isolated from lymph nodes of immunized mice were refractory to restimulation with MOG 35–55 peptide in vitro and maintained more of a naive phenotype than WT counterparts. The resistance of Kv1.3 KO mice to EAE was also associated with decreased production of IFN-γ and IL-17 by CD4+ T cells and fewer encephalitogenic T cells in the brains of affected animals. Quiescent mouse T cells express fewer Kv channels than human T cells (20 versus 250/cell) and multiple Kv family members—Kv1,1, Kv1,2, Kv1,3, Kv1,6, and Kv3,1—in contrast to human T cells that only express Kv1,3 (1). Because of these differences, it is widely believed that mouse T cells are less sensitive to blockade or absence of Kv1,3 (1, 35, 36). In addition, it has been suggested that murine T cells differ from human T cells in maintaining high levels of KCa3.1 upon acquiring an effector memory phenotype, again making them less sensitive to Kv1,3 blockade (1, 35). Our results demonstrate that Kv1,3 is the only Kv channel in murine MOG 35–55-restimulated CD4+ T blasts, and these cells from Kv1,3 KO mice exhibit a complete absence of Kv current. Moreover, the absence of Kv current prevents the mice from mounting an MOG-specific EAE response. The likely presence of other channels in these cells, chloride channels previously described in thymocytes from Kv1,3 KO mice (22) and calcium-activated KCa3.1 channels (35, 37), does not appear to be sufficient to induce EAE. Perhaps Kv1,3 channels play important hitherto undefined roles by virtue of their participation in signaling complexes in T cells (1), besides their well-accepted role in the regulation of membrane potential and calcium signaling. Moreover, the availability of these mice has allowed us to gain insight into how blockade of Kv1,3 could affect the ability of CD4+ T cells to differentiate into pathogenic Th1 or Th17 cells. In addition, our data do not contradict initial findings in the Kv1,3 KO mouse, but rather extend these findings to suggest that although the development of the mouse is grossly normal, an immunological phenotype is present in effector T cells when the mouse is challenged in an Ag-specific manner.

Because Kv1,3 is known to be expressed on a variety of cell types, including APCs (25, 26), it was important to determine the contribution of APCs from Kv1,3 KO mice to the activation state of the CD4+ T cells in our experiments. To this end, we isolated CD4+ T cells from WT and Kv1,3 KO CD45.2 mice and transferred these cells into CD45.1 congenic hosts containing WT APCs. These recipients were then immunized with MOG 35–55 peptide to induce EAE, and the ability of the WT and Kv1,3 KO CD4+ T cells to proliferate in the context of WT APCs was compared using flow cytometry. It was difficult to detect an abundance of transferred cells, probably due to the low precursor frequency of MOG 35–55-specific cells among only 5 million transferred CD45.2 cells. However, the population of CD45.2 CD4+ T cells was significantly larger in MOG-immunized mice receiving WT CD4+ T cells than Kv1,3 KO CD4+ T cells. We were unable to detect any transferred T cells, WT or Kv1,3 KO, in unimmunized recipients 7 d after transfer (data not shown), indicating that expansion was of MOG 35–55-specific CD4+ T cells and a consequence of MOG immunization. These data suggest that whereas the ability of Kv1,3-deficient APCs to present Ag to T cells in vivo may be impaired, there is an intrinsic defect in the T cells themselves, which is preventing them from responding to a properly presented Ag. These data were confirmed using in vitro assays in which CD4+ T cells were isolated and their ability to progress through the cell cycle and proliferate in response to anti-CD3/CD28 bead stimulation was measured using BrdU incorporation and a CFSE assay. We have published recently that human T EM CD4+ T cells transduced with a dominant-negative Kv1,3 construct display a delay in transition from the G2/M to the S phase of the cell cycle. This change correlated with an increase in phosphorylation of SMAD3 and p21 expression and likely contributes to the decreased proliferative capacity of the KvDN-transduced cells (7). Similarly, we found an increase in Kv1,3 KO CD4+ T cells in G2/M phase and a decrease in S phase compared with WT controls. These data suggest that cell cycle delay might contribute to the decreased proliferative capacity of CD4+ T cells from Kv1,3 KO mice. Moreover, a CFSE assay showed that proliferation of Kv1,3 KO CD4+ T cells was delayed but not completely impaired compared with WT controls fol-
lowing in vitro stimulation with anti-CD3/CD28–coated beads. This ability of Kv1.3 KO CD4+ T cells to proliferate partially in vitro may be a result of overly robust anti-CD3/CD28 stimulation or evidence that deletion of Kv1.3 in APCs contributes to the more pronounced defect in CD4+ T cell activation observed in vivo. However, DCs from Kv1.3 KO mice activate WT CD4+ T cells and produce IL-6 as effectively as WT DCs, highlighting the importance of the T cell defect. Furthermore, it was not an increase in apoptosis of Kv1.3 KO CD4+ T cells that was responsible for the observed decreased proliferative capacity. Collectively, our results suggest that the Kv1.3 KO CD4+ T cells are unable to progress through the cell cycle and become fully activated because of a defect in cell signaling or because the cells differentiate into a subset with a lower proliferative capacity.

Tregs exhibit a lower proliferative capacity and produce more IL-10 and less IL-17 and IFN-γ than other types of effector T cells (28). Therefore, it is possible that the Kv1.3 KO CD4+ T cells in MOG-immunized mice are a type of regulatory cell. Our data show, for the first time, to our knowledge, that CD4+ T cells from MOG-immunized mice have a low proliferative capacity and inhibit proliferation of WT CD4+ T cells to an extent that animals deficient in Kv1.3 may acquire more natural Tregs because of a defect in cell signaling or because the cells are representative of an IL-10+Foxp3+ T cell subset that develops in MOG-immunized Kv1.3 KO mice.

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We published recently that dominant-negative suppression of Kv1.3 expression in human CD4+ Tregs leads to phosphorylated SMAD3-mediated cell-cycle delay (7). Interestingly, phosphorylated SMAD3 accumulates in the nuclei of IL-10–secreting Kv1.3 KO CD4+ T cells with suppressor activity. TGF-β–induced SMAD 2/3 phosphorylation is followed by nuclear translocation and phosphorylation of the transcription factor SMAD3 (34). Therefore, depletion of intracellular calcium and the resulting inhibition of the calcium-calmodulin II kinase, as may occur in the absence of Kv1.3, may activate the SMAD complex (41, 42) and lead directly to increased IL-10 production and the nonclassical Treg subset that develops in MOG-immunized Kv1.3 KO mice.

Treg cells with the capacity to suppress unwanted immune responses. The data presented in this study demonstrate the importance of the Kv1.3 channel for the activation of CD4+ T lymphocytes in an Ag-specific mouse model of inflammation/autoimmunity. CD4+ T cells from Kv1.3-deficient mice exhibit a decreased overall proliferative capacity that is, at least in part, due to an intrinsic defect in the T cells themselves. This decreased proliferative capacity is associated with the development of T cells with regulatory properties and results in a significantly decreased susceptibility to EAE. Further investigation into the downstream pathways that are directly affected by Kv1.3 deficiency and the molecular mechanism by which these pathways are altered could provide new insight into the development of novel therapies focused on the enhancement of Treg function. Overall, these data suggest that specific and potent blockade of Kv1.3 could be beneficial for the treatment of immune-mediated inflammatory diseases such as MS.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. The phenotype of CD4^+ T cells from unimmunized Kv1.3 KO mice does not differ from that of WT controls. A) Thymocytes were isolated from 8-10 week old unimmunized Kv1.3 KO or WT mice, stained directly ex-vivo for CD4 and CD8 and analyzed by flow cytometry. There was no difference in the numbers of CD4^+, CD8^+, or CD4^+CD8^+ thymocytes from Kv1.3 KO mice compared to WT controls. B), C), D), and E) Spleens and lymph nodes were isolated from 8-10 week old unimmunized Kv1.3 KO or WT mice and stained directly ex-vivo for CD3, CD4, CD8, CD62L, CD44, IFN-γ, IL-17, IL-10, and Foxp3. Cells were gated on live CD3^+ (B) or CD3^+CD4^+ (C-E) and analyzed using flow cytometry. No differences were seen between Kv1.3 KO and WT mice. F) CD4^+ T cells were isolated from unimmunized Kv1.3 KO or WT mice and used in an in vitro suppression assay with CFSE labeled WT CD4^+ responder T cells. No suppression of proliferation was seen with either Kv1.3 KO or WT T cells.
Supplemental Figure 2. Gocke et al. 2012

Supplemental Figure 2. **CD4^+ T cells were gated on Foxp3^- cells prior to analysis of CD25 expression.** A) Lymph nodes were isolated from Kv1.3 KO or WT mice induced to develop EAE 7 days post-immunization and stained directly ex-vivo for CD4, Foxp3, and CD25. Cells were gated on CD4^+ Foxp3^- prior to analysis of CD25 expression.