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

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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: 5877–5886.

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 formed by 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 glomerulonephritis (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
differentiation and resulting phenotype of T cells during an inflammatory immune response.

Murine T cells have a very different ion channel expression pattern, both in number and type of channel expressed (1, 18–21). Quiescent mouse CD4+ T cells express 10–20 voltage-gated Kv channels/cell, which include Kv1.1, Kv1.2, Kv1.3, and Kv1.6 channels, whereas resting mouse CD8+ T cells express 20–35 Kv.3.1 channels/cell (18–21). Activation of these subsets results in a 6–10-fold increase in the amplitude of the Kv current, which appears to be exclusively Kv1.3 channels (1, 20, 21). An original study describing the generation of the Kv1.3 knockout (KO) mouse indicated no significant role for Kv1.3 in the development of the immune system (22). Kv1.3 KO CD4+CD8+ thymocytes expressed mRNAs and/or proteins for several other K+ channels, including Kv1.1, Kv1.4, Kv1.6, Kv2.1, Kv3.1, and KCa2.2, and they also exhibited a compensatory upregulation of anion currents with properties resembling CIC-3 (22). The immune cells of these mice developed normally with similar numbers of T lymphocytes in the spleen and thymus (22). There was no evidence of a global impact on T cells, as no significant difference was noted in the proliferation of splenocytes from Kv1.3 KO mice in response to Con A or anti-CD3 (22).

As the generation of naive T cells appears to proceed normally, we wanted to determine the role of Kv1.3 in the development of a chronic autoimmune response. Therefore, we compared the susceptibility of Kv1.3-deficient and wild-type (WT) mice to the induction of experimental autoimmune encephalomyelitis (EAE) 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, 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, C3H/HeN, 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) was administered at the time of immunization and 48 h later by i.p. injection. Mouse 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% FBS (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 × 10^5/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 × 10^3 irradiated APCs/well. To assess Ag-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 then stained 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 as 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 5 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 × 10^6/ml and stimulated with 5 µM 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 FACScalibur flow cytometer (BD Biosciences) to assess cell division. The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

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 stained 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 1 h with DNAase at 37 °C 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 × 10^6/ml and stimulated with 5 µM 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 FACScalibur 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-clamped using whole-cell configuration (T). Only activated T cells were chosen by selecting for cells with membrane capacitances >4 pF (cell diameter >11 μm). Kv1.3 currents were recorded in normal Ringer solution with a Cs+2-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, Vε11 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 eBioscience, 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 (CD44+CD62L+), TCM (CD44+CD62L−), TCM (CD44−CD62L+), and activated T (CD25+Foxp3−) cells in unimmunized and immunized mice by flow cytometry directly ex vivo. In unimmunized animals, Kv1.3 KO and WT mice were found to have similar percentages of CD4+, CD8+, and 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 TEx 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 TEx 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 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). Nevertheless, some CD4+ splenic T cells isolated from these mice did undergo activation based on cell size (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 by flow cytometry (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 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
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 T cell response. As a result, the presence of CD4⁺ T cells in the Kv1.3 KO mice contributes to their ability to suppress autoreactive effector 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 defect in CD4⁺ T cells from Kv1.3 KO mice is due to an inability to suppress autoreactive effector T cells.

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. Cultures were pulsed with 5 μg/ml of MOG 35–55 peptide, plated with 2D2 TCR-transgenic cells at different ratios, and 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.

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 × 10⁶ 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.

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 (Treg). 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.
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 Tregs.

We confirmed enhanced IL-10 secretion by CD4+ T cells from Kv1.3 KO mice independent of effects from APCs. CD4+ T cells from immunized 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.

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 Kv1.1X 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 Tregs (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 Tregs (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
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 associated with decreased proliferation of CD4+ T cells in vitro and ex vivo. (A) CD4+ T cells were isolated from unimmunized Kv1.3 KO and WT mice. Cells were stimulated in vitro with anti-CD3/CD28 beads for three rounds. Six hours following the third stimulation, cells were harvested, cell lysates were prepared, and phospho-SMAD3, SMAD3, and actin were measured by Western blotting. One representative blot is shown. (B) Percent of maximum average densitometric ratio relative to actin was calculated for three independent experiments and is shown on a bar graph.

We used Kv1.3 KO mice to determine the specific 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 TEm 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-
loving in vitro stimulation with anti-CD3/CD28-coated beads. This ability of Kv1.3KO 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.

Tₐreg 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 Kv1.3 KO mice acquire regulatory properties that could contribute to their resistance to EAE. Directly ex vivo, at 14 d postimmunization, CD4⁺ T cells from Kv1.3 KO mice secreted more IL-10 than WT cells. In addition, after multiple rounds of stimulation in vitro, Kv1.3 KO CD4⁺ T cells secreted more IL-10 than WT cells. Moreover, Kv1.3 KO CD4⁺ T cells from MOG-immunized mice or generated by multiple rounds of in vitro stimulation significantly suppressed the proliferation of WT CD4⁺ T cells. Interestingly, these Kv1.3 KO IL-10–producing CD4⁺ T cells with regulatory activity are not Foxp3⁺, suggesting that they may be representative of an IL-10⁺Foxp3⁻ regulatory subset that is reported to arise following antigenic stimulation in vitro and in vivo (38). A more thorough analysis of the IL-10 secreting Kv1.3 KO CD4⁺ T cells needs to be conducted to determine if they acquire additional markers of Tₐreg, including CTLA-4, or if they represent a distinct population of CD4⁺ T cells with regulatory characteristics that differ from canonical Tₐreg, such as naturally present IL-10–secreting Tₐreg subsets (CD4⁺CD25⁺LAP⁺, CD4⁺NK2G2D⁺, CD4⁺IL-7R⁺, CD4⁺CD25⁺LAG3⁺) (39, 40).

We published recently that dominant-negative suppression of Kv1.3 expression in human CD4⁺ Tₐreg cells 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 association with SMAD4, which induces transcription of IL-10 (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 noncanonical Tₐreg subset that develops in MOG-immunized Kv1.3 KO mice.

Taken together, these data suggest that in the absence of Kv1.3, antigenic stimulation induces differentiation of CD4⁺ T cells into a unique population of Ag-specific Tₐregs rather than into effector cells. Immunoregulatory CD4⁺CD25⁺ T cells that secrete IL-10 have been demonstrated to play an important role in peripheral tolerance to self-Ags in mice and humans (43). Naturally occurring Tₐreg have a low proliferative capacity and inhibit proliferation of CD4⁺CD25⁺ T cells (44). CD4⁺ T cells from Kv1.3 KO mice display both of the aforementioned characteristics, suggesting that animals deficient in Kv1.3 may acquire more natural Tₐreg during development. Tₐreg have been explored for therapeutic purposes for a number of inflammatory diseases (45–47), and our data suggest that pharmacological blockade or genetic suppression of Kv1.3 could provide a novel approach to generate Ag-specific Tₐreg with the capacity to suppress unwanted immune responses.

The data presented in this study demonstrate the importance of the K⁺ channel Kv1.3 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 suscepti-

bility 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 Tₐreg. 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 interests of interest.

References


