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Antigenic Stimulation of Kv1.3-Deficient Th Cells Gives Rise to a Population of Foxp3-Independent T Cells with Suppressive Properties

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Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the CNS that has been linked with defects in regulatory T cell function. Therefore, strategies to selectively target pathogenic cells via enhanced regulatory T cell activity may provide therapeutic benefit. Kv1.3 is a voltage-gated potassium channel expressed on myelin-reactive T cells from MS patients. Kv1.3-knockout (KO) mice are protected from experimental autoimmune encephalomyelitis, an animal model of MS, and Kv1.3-KO Th cells display suppressive capacity associated with increased IL-10. In this article, we demonstrate that myelin oligodendrocyte glycoprotein–specific Kv1.3-KO Th cells exhibit a unique regulatory phenotype characterized by high CD25, CTLA4, pSTAT5, FoxO1, and GATA1 expression without a corresponding increase in Foxp3. These phenotypic changes result from increased signaling through IL-2R. Moreover, myelin oligodendrocyte glycoprotein–specific Kv1.3-KO Th cells can ameliorate experimental autoimmune encephalomyelitis following transfer to wild-type recipients in a manner that is partially dependent on IL-2R and STAT5 signaling. The present study identifies a population of Foxp3− T cells with suppressive properties that arises in the absence of Kv1.3 and enhances the understanding of the molecular mechanism by which these cells are generated. This increased understanding could contribute to the development of novel therapies for MS patients that promote heightened immune regulation.

Th cells are able to ameliorate EAE induction in vivo, at least in part, via an IL-2R–dependent mechanism. Furthermore, unlike canonical Tregs, Kv1.3-KO Th cells secrete large amounts of IL-2 in vitro, which may render them a uniquely self-sustaining regulatory Th cell population. Further study of Kv1.3-KO Th cells could elucidate novel mechanisms or pathways to enhance regulation of immune responses in immune-mediated diseases, such as MS.

Materials and Methods

**Mice**

C57BL/6 (CD45.2+) and CD45.1+ congenic mice were purchased from the National Cancer Institute (Frederick, MD). Mice with a transgenic TCR specific for MOG 35–55 (2D2 mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). 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. Kv1.3-KO mice expressing MOG-specific TCR (2D2-Kv1.3 KO) were generated and bred in our facility. Mice of 8–12 wk of age were used in all of the experiments. Age-, sex-, and genotype-matched mice were used in all experiments as controls.

**Induction of EAE**

For EAE induction, mice were immunized s.c. over two sites on the lateral abdomen with 100 μg MOG 35–55 peptide (The Johns Hopkins Peptide Synthesis Core Facility, Baltimore, MD) in CFA containing 4 μg/ml Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI) followed by i.p. injections of 250 ng Bordetella pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2 after immunization. Mice were scored daily for disease progression using the following scale: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, quadriplegia or moribund; and 5, death due to EAE.

**In vivo suppression assay**

For the in vivo suppression assay, 2D2 wild-type (WT) and 2D2-Kv1.3–KO Th cells were cultured with irradiated WT splenocytes and 10 μg/ml MOG 35–55 for 72 h. Ficoll gradient centrifugation was used to remove dead cells, resulting in >90% pure CD4+ T cells, and 5 × 10^5 cells were transferred i.p. into CD45.1+ congenic recipients 1 d prior to immunization. One day after transfer, the recipient mice were immunized to induce EAE, as described above.

**CFSE proliferation assay**

Following isolation, 2D2-Kv1.3–KO 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 × 10^6/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 the 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 10 μg/ml MOG 35–55 and irradiated APCs in the presence or absence of 20 μg/ml IL-2. The cells were cultured for 72 h, stained with an anti-CD4–allophycocyanin Ab (BD Biosciences, San Jose, CA), and run on the FACS Calibur flow cytometer to assess cell division. The data were analyzed using FlowJo software (TreeStar, Ashland, OR).

**In vitro suppression assay**

CD4+ T cells were isolated from 2D2-Kv1.3–KO or 2D2 WT mice and stimulated with 10 μg/ml MOG 35–55 and irradiated APCs in the presence of 10 ng/ml IL-12, 20 ng/ml anti–IL-10, 20 μg/ml anti–CTLA-4, or isotype control Ab. Immediately preceding restimulation, Kv1.3-KO or WT T cells were cotransferred with WT naive CD4+ CFSE-labeled responder cells at a ratio of 5:1, respectively. The cells were stimulated with anti-CD3/CD28 beads for an additional 72 h, and proliferation of responder cells was assessed using a FACS Calibur flow cytometer. Data were analyzed using FlowJo software.

**Perfusion and immunostaining**

EAE mice receiving 2D2-Kv1.3–KO or 2D2 WT Th cells were anesthetized and perfused with HBSS, and blood, lymph nodes, spleens, and brains were removed. CNS tissue was digested and enriched for mononuclear cells. Single-cell suspensions were made from blood, lymph nodes, and spleens, as described below. Collected cells were stimulated in media containing PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (1 μg/ml; Sigma-Aldrich), and monensin (4 μM/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–CD4 conjugated to (BD Biosciences; RM4-5) Percep or allophycocyanin, anti–CD45.2 FITC, anti–CD11b allophycocyanin, and anti-Ly6G PE (BD Biosciences and eBioscience, San Diego, CA). Flow cytometry was performed using a FACS Calibur flow cytometer, and data were analyzed using FlowJo software (TreeStar).

**Cell culture and polarization**

Spleens and lymph nodes were isolated from immunized or naive mice, and single-cell suspensions were made by passing through a 70-μm nylon cell strainer (BD Biosciences). Th cells were isolated from splenocytes by negative selection using an EasySep Mouse CD4+ T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada), following the manufacturer’s protocol. Cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% v/v FBS (Life Technologies), 100 μg/ml penicillin and streptomycin (Quality Biological, Gaithersburg, MD), 0.5 μM 2-ME (Life Technologies), 10 mM HEPES buffer (Quality Biological), 1 mM sodium pyruvate (Sigma-Aldrich), and MEM Non-Essential Amino Acid Solution (Sigma-Aldrich). For polarization to Th1, cells were cultured in complete RPMI 1640 in the presence of 10 ng/ml IL-12. For polarization to Tregs, cells were cultured in complete RPMI 1640 in the presence of 5 ng/ml TGF-β. For polarization to Th17, cells were cultured in IMDM-based medium in the presence of 20 ng/ml IL-6, 5 ng/ml TGF-β, and 20 ng/ml IL-23. For polarization to Th2, cells were cultured in complete RPMI 1640 in the presence of 20 ng/ml IL-4. Th0 cells were cultured in complete RPMI 1640 without polarizing cytokines. For blockade experiments, cells were cultured in the presence of 10 ng/ml anti–IL-2, 10 μg/ml anti–IL-10, 20 μg/ml anti–CTLA-4, or isotype control Ab prior to stimulation with MOG 35–55 peptide. Exogenous IL-2 was used at 20 ng/ml.

**Flow cytometry**

For intracellular cytokine staining, cells were stimulated with Cell Stimulation Cocktail plus protein transport inhibitors (eBioscience) for 4–6 h. Surface Ags were stained with the following Abs: anti–CD4 (BD Biosciences; RM4-5), anti–CD44 (BD Biosciences; IM7), anti–CD2L2 (eBioscience; MEL-14), anti–CD45.1, and anti–CD25 (BD Biosciences; 7D4). For intracellular staining, cells were fixed and permeabilized with the Foxp3 staining buffer kit (eBioscience) and stained for intracellular cytokines and proteins with anti–IFN-γ (BD Biosciences; XMG1.2), anti–IL-17 (BioLegend; TC11-18H10.1), anti–CTLA-4, and anti–Foxp3 (eBioscience; FJK-16s) Abs. Flow cytometric analyses were performed on a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

**Calcium flux**

CD4+ T cells were cultured in complete RPMI 1640 and activated with anti–CD3/CD28–coated beads. The cells were either examined immediately ex vivo or after 72 h of stimulation using a flow cytometric assay. To examine Ca2+ flux, the cells were loaded with the Ca2+ indicator dyes Fluo-4 and Fura Red, surface stained for CD4, and suspended in RPMI 1640. Intracellular Ca2+ amounts were plotted as a median ratio of Fluo-4/Fura Red over time to minimize the variability in dye loading. To induce TCR signaling, CD4+ T cells were precoated with biotinylated anti-CD3e Ab and treated with streptavidin.

**ELISA**

To quantify cytokine production, supernatants from cultured cells were collected after 72 h, and the levels of IL-2, IFN-γ, IL-17, IL-10, and IL-4 were quantified using ELISA kits, according to the manufacturer’s instructions (BioLegend).

**Western blotting**

For whole-cell protein extracts, cells were collected and lysed with RIPA buffer (Boston BioProducts, Ashland, MA). Protein was quantitated using the BCA assay (Pierce, Rockford, IL). Lysates were electrophoretically separated using 12% Tris-HCl gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Western blotting was performed using Abs specific for the following proteins: pSTAT5, STAT5, pAkt, Akt, p56lck, phospho-p56lck, pErk1/2, Erk1/2, pFoxO1/FoxO3a, FoxO1, GATA-1 (all from Cell Signaling Technologies, Danvers, MA), NFATc1, goat anti-rabbit
IgG-HRP, and goat anti-mouse IgG-HRP. The average densitometric ratio was calculated for each blot by normalizing to an actin loading control using ImageJ software.

**Statistical analysis**

Statistical analysis was conducted using GraphPad Prism software (GraphPad, San Diego, CA). The Mann–Whitney U test was used to compare data from noninterval scales (e.g., clinical EAE scores). The two-tailed Student t test was used to analyze normally distributed data. Results were considered significant at \( p < 0.05 \). Error bars represent SEM in all figures.

**Study approval**

All mice were maintained in a federally approved animal facility at The Johns Hopkins University in accordance with guidelines and approval from the Institutional Animal Care and Use Committee.

**Results**

**MOG-specific Kv1.3-KO Th cells express significantly more CD25 and CTLA4 but less Foxp3 than WT cells**

Ag-specific Tregs have the potential to eliminate pathogenic autoreactive cells within the target organ more efficiently than polyclonal Tregs (8). To determine whether Kv1.3-KO Th cells with specificity for myelin Ags retain the regulatory properties observed in Th cells from the Kv1.3-KO EAE animals (17), we crossed the Kv1.3-KO mouse to the 2D2 TCR-transgenic mouse that expresses the V\(_{\alpha}3.2/V\beta11 TCR specific for MOG 35–55 peptide (18). Directly ex vivo, 2D2 WT and 2D2-Kv1.3–KO Th cells isolated from naive animals exhibited equivalent expression of CD44, CD62L, CD25, CTLA4, and Foxp3 (Fig. 1A, 1C). Following stimulation with MOG 35–55 in the presence of irradiated WT APCs (or KO APCs), 2D2 WT and 2D2-Kv1.3–KO Th cells expressed equivalent levels of the activation markers CD44 and CD62L, suggesting that the T cells from 2D2-Kv1.3–KO mice do not display a different threshold for activation compared with WT T cells. However, 2D2-Kv1.3–KO Th cells expressed significantly higher levels of CD25 than did the 2D2 WT cells (Fig. 1B, 1D). Interestingly, this CD25\(^{hi}\) population of cells was not Foxp3\(^{+}\) but did exhibit increased expression of CTLA4, a molecule implicated in canonical Treg function. These data indicate that Ag-specific Kv1.3-KO Th cells have a phenotype consistent with the suppressive phenotype observed in the Kv1.3-KO EAE animals.

**2D2-Kv1.3–KO Th cells secrete significantly more IL-10, IL-4, and IL-2 and significantly less IFN-\(\gamma\) and IL-17**

It has been well described in the literature that Th cells of the Th1 and Th17 lineages are detrimental in MS (19–24). Much effort has been focused on deviating the phenotype of the pathogenic T cells away from Th1 and Th17 and toward Th2 and Tregs (25–29). Moreover, Ag-specific Tregs have the ability to suppress the production of IFN-\(\gamma\) and IL-17 from myelin-reactive T cells (8, 30). Further examination revealed that 2D2-Kv1.3–KO Th cells stimulated with MOG in vitro secretes increased amounts of IL-4 and IL-10 and significantly less of the proinflammatory cytokines IFN-\(\gamma\) and IL-17 than did 2D2 WT cells, consistent with deviation toward a regulatory or suppressive phenotype (Fig. 2A). Importantly, 2D2-Kv1.3–KO Th cells also secreted significantly more IL-2 than did WT controls (Fig. 2B) and displayed an enhanced proliferative response to IL-2 (Fig. 2C), indicating that, unlike conventional Tregs, Kv1.3-KO Th cells may be able to sustain their own proliferation without exogenous IL-2.

**Antigenic stimulation of 2D2-Kv1.3–KO Th cells results in increased phosphorylation of STAT5 and decreased phosphorylation of p56lck and Erk1/2**

To further characterize the signaling pathway that culminates in increased CD25 and CTLA4 expression and IL-4, IL-10, and IL-2 production by Kv1.3-KO Th cells, we sought to examine TCR and IL-2R signaling following antigenic stimulation of 2D2 WT and 2D2-Kv1.3–KO Th cells. Expression and phosphorylation of STAT5, p56lck, Akt, and Erk in 2D2 WT and 2D2-Kv1.3–KO Th cells were examined directly ex vivo or following 72 h of stimulation with MOG 35–55 and irradiated WT APCs. Interestingly, we found significantly increased phosphorylation of STAT5 in 2D2-Kv1.3–KO Th cells compared with 2D2 WT controls (Fig. 2D). This finding correlated with a slight decrease in phosphorylation of p56lck and a significant decrease in phosphorylation of Erk1/2 (Fig. 2D, 2E). No significant changes in phosphorylation of Akt were observed. These data suggest that absence of Kv1.3 results in decreased signaling through the TCR and increased signaling through the IL-2R/STAT5 pathway. Importantly, these increases in pSTAT5 were observed in 2D2-Kv1.3–KO T cells, regardless of whether cells were stimulated in the presence of WT or Kv1.3–KO APCs (Supplemental Fig. 1), indicating that the Th cell–suppressive phenotype is due to T cell–intrinsic signaling events.

The regulatory phenotype of Kv1.3-KO Th cells is not a result of depletion of intracellular calcium or changes in NFAT signaling

Kv1.3 plays a fundamental role in the activation of T cells through regulation of cytoplasmic free calcium levels (12). Thus, we sought to investigate whether the regulatory phenotype of Kv1.3-KO Th cells associated with decreased proximal TCR signaling could be attributed to defective calcium signaling, which could lead to the development of anergic T cells with suppressive capabilities. To this end, calcium flux induced by TCR cross-linking was evaluated in Kv1.3-KO and WT Th cells either directly ex vivo or...
following 72 h of stimulation using a flow cytometric method. Surprisingly, no difference was observed in calcium flux in Kv1.3-KO T cells compared with WT controls (Fig. 3A). To confirm that there is no defect in intracellular calcium signaling in Kv1.3-KO Th cells, Th cells were stimulated in vitro for 24 h, and expression and nuclear translocation of the calcium sensitive transcription factor NFAT were evaluated by Western blotting. No difference in NFAT activation or translocation was observed, confirming that intracellular calcium signaling is unaffected in the absence of Kv1.3 (Fig. 3B), likely due to compensation from other potassium or chloride channels (31). These data highlight an important role for Kv1.3 in TCR signaling that is independent of its role as an ion channel.

**FoxO1 nuclear localization and GATA1 expression are increased following stimulation of Kv1.3-KO Th cells in vitro**

FoxO transcription factors were demonstrated to regulate cell cycle progression, survival, and homeostasis (32, 33). Under conditions of strong TCR stimulation, FoxO proteins are phosphorylated by a variety of molecules, including Akt, Erk, and Sgk1, and shuttled from the nucleus to the cytoplasm, where they may be targeted for degradation (34). FoxO proteins were demonstrated to control immune homeostasis and the function of Tregs via unique transcriptional programs that are not dependent on Foxp3 (35). Moreover, FoxO1 was shown to regulate transcription of CTLA4 directly and to repress IFN-γ expression in Tregs (33, 35). To investigate whether FoxO1 expression was increased in Kv1.3-KO Th cells and, thus, could be inducing or repressing transcription of regulatory or inflammatory genes, respectively, we examined the phosphorylation and nuclear translocation of FoxO1 in Kv1.3-KO, WT, 2D2-Kv1.3–KO, and 2D2 WT Th cells. We observed a significant increase in nuclear FoxO1 expression, which correlated directly with decreased phosphorylation of the protein in 2D2-Kv1.3–KO T cells, as well as Kv1.3-KO non-TCR–transgenic T cells (Fig. 4A, 4B). Moreover, it was recently described that the transcriptional signature of Tregs is made up of multiple transcription factors that act in a redundant fashion (36). In addition to Foxp3, GATA1 is one of the transcription factors that makes up this signature. Because Kv1.3-KO T cells do not express increased Foxp3, we investigated the possibility that one or more of the other Treg signature molecules plays a role in promoting the regulatory phenotype observed. To this end, CD4+ T cells from Kv1.3-KO or WT mice were stimulated in vitro, and GATA1 expression was examined by Western blotting. Interestingly, Kv1.3-KO T cells consistently express significantly more GATA1 than do WT T cells (Fig. 4C), supporting a novel regulatory role for GATA1 not only in lymphocytes, but specifically in Kv1.3-deficient T cells.

**Kv1.3-KO Th cells maintain a stable phenotype in a highly inflammatory environment and consistently express increased FoxO1 and GATA1 transcription factors**

Next, we sought to assess whether the regulatory phenotype of Kv1.3-KO Th cells is maintained in the face of large amounts of proinflammatory cytokines, as would occur during immune-mediated diseases, such as MS. To this end, we polarized WT and Kv1.3-KO Th cells to Th0, Th1, Th2, Th17, and Treg subtypes and examined the expression of signature cytokines, as well as pSTAT5, FoxO1, and GATA1, under these conditions. When polarized to Th1 or Th17, Kv1.3-KO Th cells produced significantly less IFN-γ and IL-17, respectively, than did the WT Th cells (Fig. 5A, 5B). Importantly, under all polarization conditions, Kv1.3-KO Th cells expressed significantly more pSTAT5, less pFoxO1, and more GATA1 than did WT controls (Fig. 5C). Additionally, Kv1.3-KO or WT CD4+ T cells were stimulated for

**FIGURE 2.** 2D2-Kv1.3–KO Th cells display increased IL-10, IL-4, and IL-2 secretion and increased expression of pSTAT5 accompanied by decreased IFN-γ and IL-17 secretion and decreased phosphorylation of p56lk and Erk1/2. 2D2 WT and 2D2-Kv1.3–KO Th cells were stimulated with MOG 35–55 and irradiated APCs for 0 or 72 h. Production of IFN-γ, IL-17, IL-4, and IL-10 (A) and IL-2 (B) and Akt and Erk1/2 (D) was analyzed by ELISA. (C) 2D2-Kv1.3–KO Th cells were labeled with CFSE and stimulated with 10 μg/ml MOG 35–55 and irradiated APCs for 72 h in the presence or absence of 20 ng/ml RIL-2. CFSE dilution in CD4+ T cells was analyzed by FACS analysis. The percentage of undivided cells is shown in the line graphs. STAT5, p56lk, and β-actin (D) and Akt and Erk1/2 (E) expression and phosphorylation were determined using Western blotting. Numbers below the bands indicate average densitometric ratio normalized to total protein (for phospho blots) or actin loading control. **p < 0.01, ***p < 0.001.

**FIGURE 3.** Kv1.3 is not required for TCR cross-linking–induced elevation in cytoplasmic free Ca2+ by naive or effector T cells. (A) WT (red line) and Kv1.3-KO (blue line) Th cells were loaded with the Ca2+ indicator dyes Fluo-4 and Fura Red, surface stained for CD4, and suspended in RPMI 1640 either directly ex vivo or 72 h after stimulation with anti-CD3/CD28–coated beads. Th cells were precoated with biotinylated anti-CD3ε Ab and treated with streptavidin (TCR) at the indicated time (arrow). Intracellular Ca2+ amounts were plotted as a median ratio of Fluo-4/Fura Red concentration dyes over time to minimize the variability in dye loading. (B) WT and Kv1.3-KO Th cells were stimulated with anti-CD3/CD28 beads for 24 h. Nuclear and cytoplasmic extracts were prepared, and NFATc1 expression and nuclear translocation were determined using Western blotting. Numbers below the bands indicate average densitometric ratio normalized to actin loading control.
Foxp3+ Tregs were demonstrated to reduce disease severity. However, although the percentage of CD11b+ cells was increased in the mice receiving WT T cells, likely due to more infiltrating monocytes in EAE animals, we saw no differences in the percentages of CD11b+Ly6G+ cells in the brains of mice receiving Kv1.3-KO Th cells at the peak of disease compared with mice receiving WT Th cells (Supplemental Fig. 3C). These data suggest that induction of myeloid-derived suppressor cells is not the likely mechanism of Kv1.3-KO Th cell–mediated suppression.

Neutralization of IL-2 results in a partial loss of the regulatory phenotype in Kv1.3-KO Th cells

To determine whether 2D2-Kv1.3–KO Th cells are dependent upon IL-2 for the development of their regulatory phenotype, we neutralized IL-2 using a blocking Ab in 2D2 WT and 2D2-Kv1.3–KO Th cell cultures during a 72-h stimulation with MOG 35–55 and irradiated APCs and examined the effects on CD25, pSTAT5, and pFoxO1 expression, as well as cytokine production and suppressive function. IL-2 neutralization was confirmed by ELISA (Supplemental Fig. 4A). We observed no increase in CD25 or pSTAT5 in Kv1.3-KO cells when IL-2 was blocked (Fig. 7A, 7B) and no increase in IL-10 or IL-4 secretion (Fig. 7C), suggesting that IL-2 signaling plays an important role in the development of the regulatory phenotype observed in the absence of Kv1.3. Accordingly, when IL-2 was neutralized, the ability of Kv1.3-KO Th cells to suppress the proliferation of WT effector T cells was impaired in an in vitro suppression assay. This suppressive quality was not affected when IL-10 or CTLA-4 was inhibited, supporting an important role for IL-2 in Kv1.3-KO Th cell–mediated suppression (Supplemental Fig. 4B–D). Interestingly, 2D2-Kv1.3–KO Th cells did not express Foxp3. 2D2 WT cells also were Foxp3− (Fig. 6A). Remarkably, animals that received activated 2D2-Kv1.3–KO T cells at the time of immunization exhibited a significantly decreased incidence and severity of EAE compared with immunized animals receiving 2D2 WT T cells or no cells (Fig. 6B). This decreased disease severity could not be attributed to impaired migration of transferred Kv1.3-KO Th cells or host CD4+ T cells to the CNS, because equal numbers of CD4+ T cells were detected in the blood, lymph nodes, and spleens of mice at 7 and 14 d postimmunization (Supplemental Fig. 3A, 3B). In a parallel experiment, 2D2-Kv1.3–KO Th cells were compared with 2D2 WT Th cells polarized to a Foxp3+ Treg phenotype prior to transfer for their ability to suppress EAE. Kv1.3-KO Th cells suppressed disease equally to WT Tregs and, importantly, maintained their suppressive phenotype longer than did Foxp3+ Tregs in vivo (Fig. 6C). To explore the mechanism by which transfer of 2D2-Kv1.3–KO T cells confers protection from EAE, we investigated whether transfer of these T cells induced expansion of endogenous Tregs, which, in turn, would protect the mice from EAE. For this purpose, 2D2-Kv1.3–KO and 2D2 WT CD4+ T cells were transferred into CD45.1 congenic recipients, which were immunized with MOG peptide to induce EAE. The percentage of CD45.1– WT, CD45.1−, and Foxp3+ T cells was examined via flow cytometry at the peak of disease. No differences were detected in the percentage of host Tregs from the spleen or draining lymph nodes of mice receiving WT or Kv1.3 KO T cells, despite the significantly decreased incidence of disease in animals receiving Kv1.3 KO cells (Fig. 6D). These data have powerful implications for the ability of Foxp3− Ag-specific Kv1.3-KO Th cells to be protective in autoimmune disease via a mechanism that is not dependent on host Tregs.

We also investigated the possibility that the transfer of Kv1.3-KO Th cells resulted in the induction of myeloid-derived suppressor cells in the recipient animals, thereby contributing to the decreased disease severity. However, although the percentage of CD11b+ cells was increased in the mice receiving WT T cells, likely due to more infiltrating monocytes in EAE animals, we saw no differences in the percentages of CD11b+Ly6G+ cells in the brains of mice receiving Kv1.3-KO Th cells at the peak of disease compared with mice receiving WT Th cells (Supplemental Fig. 3C). These data suggest that induction of myeloid-derived suppressor cells is not the likely mechanism of Kv1.3-KO Th cell–mediated suppression.

Adoptive transfer of activated 2D2-Kv1.3–KO Th cells ameliorates EAE but does not induce expansion of endogenous Foxp3+ Tregs

Foxp3+ peripherally induced Tregs were demonstrated to reduce the severity of EAE when transferred at the time of immunization (37). To determine whether our Ag-specific Kv1.3-KO Th cells could reduce the severity of EAE in a similar manner, we transferred 2D2-Kv1.3–KO and 2D2 WT Th cells, which were stimulated with MOG 35–55 peptide and irradiated WT APCs for 72 h in vitro, into WT animals and then immunized the recipients to induce EAE. Prior to transfer, cells were subjected to Ficoll-density centrifugation to remove dead APCs, resulting in >90% pure CD4+ T cells. Examination of phenotype prior to transfer revealed that 2D2-Kv1.3–KO Th cells expressed significantly higher amounts of CD25 than did 2D2 WT cells but did not express Foxp3. 2D2 WT cells also were Foxp3− (Fig. 6A). Remarkably, animals that received activated 2D2-Kv1.3–KO T cells at the time of immunization exhibited a significantly decreased incidence and severity of EAE compared with immunized animals receiving 2D2 WT T cells or no cells (Fig. 6B). This decreased disease severity could not be attributed to impaired migration of transferred Kv1.3-KO Th cells or host CD4+ T cells to the CNS, because equal numbers of CD4+ T cells were detected in the blood, lymph nodes, and spleens of mice at 7 and 14 d postimmunization (Supplemental Fig. 3A, 3B). In a parallel experiment, 2D2-Kv1.3–KO Th cells were compared with 2D2 WT Th cells polarized to a Foxp3+ Treg phenotype prior to transfer for their ability to suppress EAE. Kv1.3-KO Th cells suppressed disease equally to WT Tregs and, importantly, maintained their suppressive phenotype longer than did Foxp3+ Tregs in vivo (Fig. 6C). To explore the mechanism by which transfer of 2D2-Kv1.3–KO T cells confers protection from EAE, we investigated whether transfer of these T cells induced expansion of endogenous Tregs, which, in turn, would protect the mice from EAE. For this purpose, 2D2-Kv1.3–KO and 2D2 WT CD4+ T cells were transferred into CD45.1 congenic recipients, which were immunized with MOG peptide to induce EAE. The percentage of CD45.1−, CD4+, CD25+, Foxp3+ T cells was examined via flow cytometry at the peak of disease. No differences were detected in the percentage of host Tregs from the spleen or draining lymph nodes of mice receiving WT or Kv1.3 KO T cells, despite the significantly decreased incidence of disease in animals receiving Kv1.3 KO cells (Fig. 6D). These data have powerful implications for the ability of Foxp3− Ag-specific Kv1.3-KO Th cells to be protective in autoimmune disease via a mechanism that is not dependent on host Tregs.

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Neutralization of IL-2 results in a partial loss of the regulatory phenotype in Kv1.3-KO Th cells

To determine whether 2D2-Kv1.3–KO Th cells are dependent upon IL-2 for the development of their regulatory phenotype, we neutralized IL-2 using a blocking Ab in 2D2 WT and 2D2-Kv1.3–KO Th cell cultures during a 72-h stimulation with MOG 35–55 and irradiated APCs and examined the effects on CD25, pSTAT5, and pFoxO1 expression, as well as cytokine production and suppressive function. IL-2 neutralization was confirmed by ELISA (Supplemental Fig. 4A). We observed no increase in CD25 or pSTAT5 in Kv1.3-KO cells when IL-2 was blocked (Fig. 7A, 7B) and no increase in IL-10 or IL-4 secretion (Fig. 7C), suggesting that IL-2 signaling plays an important role in the development of the regulatory phenotype observed in the absence of Kv1.3. Accordingly, when IL-2 was neutralized, the ability of Kv1.3-KO Th cells to suppress the proliferation of WT effector T cells was impaired in an in vitro suppression assay. This suppressive quality was not affected when IL-10 or CTLA-4 was inhibited, supporting an important role for IL-2 in Kv1.3-KO Th cell–mediated suppression (Supplemental Fig. 4B–D). Interestingly, 2D2-Kv1.3–KO Th cells do not have an intrinsic defect in the ability to express Foxp3

Because Kv1.3-KO Th cells have many properties common to Foxp3+ Tregs, with the exception that they do not express Foxp3, it was important to determine whether Th cells can express Foxp3 in the absence of Kv1.3. To this end, we polarized 2D2-Kv1.3-KO and WT Th cells to a Treg phenotype in vitro using TGF-β. Under polarizing conditions, Kv1.3-KO T cells can express equivalent amounts of Foxp3 as WT T cells, suggesting that Kv1.3 deficiency does not inhibit Foxp3 expression (Supplemental Fig. 2). Instead, following TCR stimulation, Kv1.3-KO Th cells appear to develop regulatory capacity independently of Foxp3, supporting the hypothesis that this population of T cells constitutes a novel subset of Th cells with suppressive properties.

Adoptive transfer of activated 2D2-Kv1.3–KO Th cells ameliorates EAE but does not induce expansion of endogenous Foxp3+ Tregs

Foxp3+ peripherally induced Tregs were demonstrated to reduce the severity of EAE when transferred at the time of immunization (37). To determine whether our Ag-specific Kv1.3-KO Th cells could reduce the severity of EAE in a similar manner, we transferred 2D2-Kv1.3–KO and 2D2 WT Th cells, which were stimulated with MOG 35–55 peptide and irradiated WT APCs for 72 h in vitro, into WT animals and then immunized the recipients to induce EAE. Prior to transfer, cells were subjected to Ficoll-density centrifugation to remove dead APCs, resulting in >90% pure CD4+ T cells. Examination of phenotype prior to transfer revealed that 2D2-Kv1.3–KO Th cells expressed significantly
T cells continued to secrete less IFN-γ and IL-17 than did WT controls, which continued to secrete significant amounts of IFN-γ and IL-17 even when IL-2 was neutralized (Fig. 7C), indicating that neutralization of IL-2 did not globally impair the function of Th cells, but rather inhibited a specific signaling pathway. Importantly, neutralization of IL-2 also did not affect FoxO1 expression, suggesting that the effect of IL-2 neutralization is not broad but rather pathway specific (Fig. 7D).

Next, we sought to determine whether IL-2 signaling and the resulting induction of pSTAT5 and CD25 are required for 2D2-Kv1.3–KO cells to ameliorate EAE. To this end, we neutralized IL-2 during the in vitro preactivation of 2D2-Kv1.3–KO and 2D2 WT T cells and then transferred these cells into WT recipients 1 d prior to immunization. Reactivation in the absence of IL-2 only partially impaired 2D2-Kv1.3–KO T cells from being able to ameliorate EAE, indicating that IL-2R signaling during antigenic stimulation contributes to the induction of a regulatory phenotype in 2D2-Kv1.3–KO Th cells (Fig. 7E).

Discussion

Impaired suppressive function of Tregs has been implicated in the immunopathogenesis of MS (3–5). In the current study, we delineated the mechanism by which a unique population of Foxp3+ Th cells develops in the absence of Kv1.3 and characterized the regulatory effects of this cell population on T cell function and disease using the EAE model. Our data suggest that CD25hi, CD44hi, IL-10–producing Foxp3+ T cells with suppressive properties are generated in the absence of Kv1.3 as a consequence of disrupted TCR/integrin signaling during formation of the immunological synapse rather than impaired voltage-gated potassium flux and corresponding changes in intracellular calcium signaling. This lack of change in intracellular calcium signaling is not completely unsurprising, because Kv1.3 is deleted during development of the mouse, thereby resulting in compensation from chloride channels, as previously reported (31). Moreover, decreased activation and phosphorylation of proximal TCR signaling molecules, including Erk1/2 and p56lck, were observed, suggesting a role for impaired TCR signaling in the development of this cell population. Not surprisingly, because phosphorylation of these proximal TCR signaling molecules is responsible for nuclear exclusion of FoxO transcription factors, the observed alteration in TCR signaling correlated with increased nuclear localization of FoxO proteins in the absence of Kv1.3. In addition to increased nuclear FoxO1 expression, increased expression of GATA1, a transcription factor thought to contribute to the Treg signature but not extensively studied in lymphocytes, was observed. The phenotype of this novel cell population remained stable in the presence of strong inflammatory conditions. Importantly, we also demonstrate that these MOG-specific noncanonical Tregs from Kv1.3-KO animals can ameliorate EAE and that this suppressive capacity is partially dependent on endogenous IL-2 signaling. Overall, our data demonstrate the development of a novel population of T cells with regulatory capacity that arise in the absence of Kv1.3 and point to FoxO1 and GATA1 as novel targets for the induction of Ag-specific Tregs during immune-mediated disease.
FIGURE 6. Adoptive transfer of activated 2D2-Kv1.3–KO Th cells ameliorates EAE but does not induce expansion of endogenous Foxp3+ Tregs. 2D2 WT and 2D2-Kv1.3–KO Th cells were stimulated with MOG 35–55 and irradiated WT APCs for 72 h. Th cells were isolated by Ficoll gradient. (A) CD25 and Foxp3 expression was evaluated by FACS analysis. (B) 2D2 WT or 2D2-Kv1.3 KO Th cells isolated after Ficoll density centrifugation were injected into WT recipients at the time of immunization with MOG 35–55. (C) Kv1.3-KO and 2D2 WT Th cells were stimulated with MOG 35–55 and irradiated WT APCs for 72 h, with or without 5 ng/ml TGF-β. Following isolation by Ficoll gradient, Th cells were transferred to WT C57BL/6 recipients at the time of immunization. Animals were monitored for EAE clinical scores. (D) 2D2 WT or 2D2-Kv1.3–KO Th cells were stimulated as described above and transferred into CD45.1 congenic recipients at the time of immunization. CD45.1+CD4+ cells from recipient lymph nodes and spleen were analyzed by FACS analysis at the peak of disease for the expansion of endogenous CD25+Foxp3+ Tregs. Bar graph is representative of five animals/group and two independent experiments.
factors identified recently to contribute to the transcriptional sig-
ificance of CD25 and/or IL-10. GATA1 was one of several transcription
factors was shown to be important for the regulatory effects of
CTLA-4, and CD25, in the absence of Kv1.3, we examined
the possibility that yet-to-be discovered populations of T cells
involved in the differentiation of Tregs. Engagement of TCR and
peptide/MHC complexes leads to the activation of a multitude of
downstream signaling molecules, including protein tyrosine
kinases and adaptor molecules, such as p56lcK, Zap70, and SLP76.
These downstream signaling events culminate in the production of
cytokines, such as IL-2 and IL-4. Binding of IL-2 to IL-2R, and to
a lesser extent, interaction of IL-4 with its receptor, play a critical
role in sustaining T cell activation and, importantly, immune ho-
meostasis. It was shown that one of the major roles of IL-2 is to
induce activation of STAT5 (39–42). STAT5 proteins were demon-
strated to be crucial for the differentiation of Tregs and Th2
cells and, indeed, deletion of STAT5 results in the development of
T cells with a hyperactivated phenotype very similar to that of the
autoimmune phenotype in IL-2β-deficient mice (40–42). Our
data demonstrate increased expression of CD25 and increased
phosphorylation of STAT5, which correlates with decreased
phosphorylation of p56lcK and Erk1/2. Kv1.3 was demonstrated to
play an important role in formation of the immunological synapse,
and p56lcK is known to interact directly with the hDlg adaptor
protein of Kv1.3, promoting signaling through the TCR during
T cell activation (43–47). Disruption of this interaction, as would
occur in the absence of Kv1.3, could result in the relocalization of
p56lcK to the IL-2R complex, thereby preferentially promoting
signaling through the IL-2R as opposed to the TCR and contrib-
uting to the differentiation of T cells with regulatory properties.
Because Erk was demonstrated to phosphorylate FoxO1 and exclu-
de it from the nucleus where it may be targeted for degradation
(34), it is possible that the reduction in Erkβ1/2 phosphorylation
that is observed in Kv1.3-KO Th cells is directly responsible for
regulation of FoxO in the absence of Kv1.3.

Importantly, we demonstrate that activated myelin-specific
Kv1.3-KO Th cells, which maintain suppressive properties in
the presence of strong inflammatory conditions, can suppress the
development of EAE when transferred to WT animals at the time of
immunization. This suppression is not dependent upon the ex-
pansion of endogenous Foxp3+ Tregs in the host, is maintained
even when Foxp3+ Treg suppression wanes, and is at least par-
tially dependent upon IL-2R signaling. The exact mechanisms by
which these cells suppress EAE in vivo, including the requirement
for IL-10, need to be addressed in future experiments. However,
these data have strong implications for this unique population of
T cells as suppressors of the aberrant immune response in MS.

Recently, a population of regulatory cells named FoxA1+ T cells
were identified in MS patients who demonstrated responsiveness to
IFN-β therapy (7). This population of cells is also Foxp3− but,
unlike the population identified in our studies, it does not secrete
increased amounts of IL-10. The identification of such a novel
population of Tregs in the context of CNS autoimmunity supports
the possibility that yet-to-be discovered populations of T cells
with regulatory properties, such as those described in this arti-
cle, exist and could arise naturally in response to disease-specific
triggers. Determining whether a natural correlate of Kv1.3-KO
Tregs exists in humans will be the focus of future studies.

Overall, our data indicate that deletion of Kv1.3 results in the
development of a unique population of Th cells via increased
STAT5 phosphorylation, IL-2R signaling, and expression of FoxO1
in these cells. In future studies, it will be important to determine
whether induction of FoxO1, GATA1, or a combination of these
two transcription factors is sufficient to convey a protective
phenotype to 2D2 WT T cells. These data highlight the potential
for the development of novel drugs that specifically induce the
expression of these transcription factors in immune-mediated
disease.

Increased IL-2 signaling through the high-affinity IL-2R con-
tributes to the differentiation of Tregs. Engagement of TCR and
peptide/MHC complexes leads to the activation of a multitude of
downstream signaling molecules, including protein tyrosine
kinases and adaptor molecules, such as p56lcK, Zap70, and SLP76.
These downstream signaling events culminate in the production of
cytokines, such as IL-2 and IL-4. Binding of IL-2 to IL-2R, and to
a lesser extent, interaction of IL-4 with its receptor, play a critical
role in sustaining T cell activation and, importantly, immune ho-
meostasis. It was shown that one of the major roles of IL-2 is to

To determine which transcription factors, in lieu of Foxp3, may
be driving the transcription of regulatory factors, such as IL-10,
CTLA-4, and CD25, in the absence of Kv1.3, we examined
FoxO family members. Our previously published data indicate that
phosphorylation of SMAD3 is increased in Kv1.3-KO Th cells
stimulated in vitro (11), and our new data demonstrate that FoxO1
expression is also increased in response to in vitro antigenic
stimulation. Synergy between SMAD and FoxO transcription
factors was shown to be important for the regulatory effects of
TGF-β in immune cells. FoxO1 plays an important role in
maintaining T cell homeostasis and induction of tolerance, and it
can dictate Treg-lineage commitment (32). FoxO1 also was im-
licated in both the repression of IFN-γ and IL-17/RORγt and in the
transactivation of genes essential for Treg function, such as
CTLA4 and Foxp3 itself (38). Our data demonstrate increased
nuclear localization of FoxO1, which correlates directly with de-
creased phosphorylation of the protein in Th cells from Kv1.3-KO
and 2D2-Kv1.3–KO mice following stimulation in vitro. We hy-
pothesized that FoxO1 could directly, or via interaction with other
transcription factors, such as GATA1, induce transcription of
CD25 and/or IL-10. GATA1 was one of several transcription
factors identified recently to contribute to the transcriptional sig-
nature of Foxp3+ Tregs (36). Because GATA1 expression was
found to be significantly increased in Kv1.3-KO T cells, it is
possible that it can compensate for the lack of Foxp3 expression in

FIGURE 7. Neutralization of IL-2 results in a partial loss of the regu-

latory phenotype in Kv1.3-KO Th cells. 2D2 WT and 2D2-Kv1.3–KO Th
cells were stimulated with MOG 35–55 and irradiated APCs for 72 h in the
presence of 10 μg/ml anti–IL-2 Ab or isotype control. CD25 and Foxp3
(A) and pSTAT5 and STAT5 (B) expression, cytokine production (C), and
FoxO1 expression (D) were examined. Numbers below the bars indicate
average densitometric ratio normalized to actin loading control. (E) Cells
described above were transferred to C57BL/6 WT recipients at the time of
immunization for EAE, and clinical score was evaluated. *p < 0.05, **p <
0.01, ***p < 0.001. ns, not significant.
and GATA1 transcription factors. Importantly, this population of Ag-specific T cells with suppressive properties arises without increased expression of Foxp3, highlighting the contribution of the aforementioned transcription factors to the signature of these cells. In addition, these results establish an in vivo model system in which to study the role of specific molecules and transcription factors, such as FoxO1, GATA1, STAT5, and IL-2, in the differentiation of this previously undescribed population of T cells. This study increases our understanding of how a novel subset of T cells may be manipulated in vivo to suppress unwanted inflammation in immune-mediated diseases in an Ag-specific manner and may pave the way for developing novel effective therapies targeted at disease-specific cells.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. 2D2-Kv1.3 KO Th cell phenotype is independent of APCs. 2D2-WT and 2D2-Kv1.3 KO Th cells were cultured with irradiated WT (top row) or Kv1.3 KO (bottom row) APCs and 10 μg/ml MOG 35-55 for 72 h. Expression of CD25 and Foxp3 (a) and pSTAT5, STAT5 and β-Actin (b) were examined by FACS analysis or western blotting respectively. The data are representative of at least 2 independent experiments. Numbers below bands indicate average densitometric ratio normalized to actin loading control.
Figure S2. 

**Kv1.3 KO Th cells polarized with TGF-β express Foxp3.** WT and Kv1.3 KO Th cells were stimulated with anti-CD3/CD28-coated beads for 72 h in the presence (Treg) or absence (Th0) of 5 ng/ml TGF-β. (a) Expression of Foxp3 and β-actin was analyzed by western blotting. Numbers below bands indicate average densitometric ratio normalized to actin loading control. (b) Expression of CD25 and Foxp3 was analyzed by FACS analysis.
Figure S3. **Kv1.3 KO Th cells are not retained in peripheral lymphoid organs and do not have more CD11b+Ly6G+ MDSCs in the brain.** 2D2-WT and 2D2-Kv1.3 KO Th cells were stimulated for 72 h *in vitro* and injected into recipients at the time of immunization. The percentage of CD4+ T cells (a) or transferred CD4+CD45.2+ T cells (b) in the blood, lymph nodes, and spleens of recipient animals were analyzed by flow cytometry at the peak of disease. Live cells were gated and percentages of CD4+ (a) and CD4+CD45.2+ (b) cells are depicted from representative animals. (c) Mononuclear cells were isolated from the brains of immunized animals at the peak of disease (day 14 post-immunization) and CD11b+Ly6G+ cells were analyzed by flow cytometry. Live cells were gated and percentages of CD11b+Ly6G+ cells are depicted from representative animals. Graphs are representative of 4 animals per group and 2 independent experiments.
Figure S4. Blockade of IL-2 decreases the suppressive capacity of Kv1.3 KO Th cells whereas blockade of IL-10 or CTLA-4 does not. CD4⁺ T cells were isolated from 2D2-Kv1.3 KO or 2D2-WT mice and stimulated with 10µg/ml MOG 35-55 +irradiated APCs in the presence of 10µg/ml anti-IL-2 (b), 10µg/ml anti-IL-10 (c), 20µg/ml anti-CTLA-4(c), or isotype control antibody and used in an in vitro suppression assay with CFSE labeled WT CD4⁺ responder T cells at a ratio of 5:1. IL-2 secretion (a) and IL-10 secretion (d) were measured by ELISA following antibody blockade at 72 hours post-activation. Cell division was analyzed by flow cytometry and percent undivided cells is depicted on the histograms. Data are representative of 2 independent experiments.