Functional Blockade of the Voltage-gated Potassium Channel Kv1.3 Mediates Reversion of T Effector to Central Memory Lymphocytes through SMAD3/p21cip1 Signaling*§

Received for publication, August 31, 2011, and in revised form, November 11, 2011. Published, JBC Papers in Press, November 22, 2011, DOI 10.1074/jbc.M111.296798

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Background: The role of Kv1.3 in regulating T cell differentiation and memory is incompletely understood. A dominant negative mutation of Kv1.3 mediates reversion of TEM into TCM through SMAD3-dependent cell cycle changes.

Results: Using a lentiviral-dominant negative approach, we show that loss of function of Kv1.3 mediates reversion of TEM into TCM. Using a lentiviral-dominant negative approach, we show that loss of function of Kv1.3 mediates reversion of TEM into TCM, via a delay in cell cycle progression at the G2/M stage. The inhibition of Kv1.3 signaling caused an up-regulation of SMAD3 phosphorylation and induction of nuclear p21cip1 with resulting suppression of Cdk1 and cyclin B1. These data highlight a novel role for Kv1.3 in T cell differentiation and memory responses, and provide further support for the therapeutic potential of Kv1.3 specific channel blockers in TEM-mediated autoimmune diseases.

Conclusion: Signalng through Kv1.3 is a mechanism by which TEM may revert to TCM.

Significance: These findings suggest a novel role for Kv1.3 in T cell differentiation and memory responses.

The adaptive immune system is characterized by the ability of lymphocytes to respond to a vast array of antigenic stimuli and then maintain recall responses to these cognate antigens for many years. The molecular mechanisms by which T cells differentiate into and maintain their status as memory cells have not been well defined, although a number of signaling pathways have been identified (1–7). After antigenic stimulation, naïve T lymphocytes clonally expand in the lymph node and differentiate into subsets of activated effector cells. These activated T cells then egress from the lymph node and home to tissue sites of inflammation where they mediate their effector functions through secretion of proinflammatory cytokines or proteases. Memory T cells are divided into two broad subsets, based on their expression of the lymph node homing chemokine receptor, CCR7, which is used to define T central memory (TCM) cells. T effector memory (TEM) cells lose CCR7 expression and thus are more able to home to tissue sites of inflammation. As T cells divide during the process of differentiation, there has been interest in understanding the coordinated process of cell cycle and T cell differentiation. The role of ion channels in regulating cell cycle was first recognized in the 1960s when it was shown that membrane voltage potentials change during the stages of cell cycle and may mediate progression through G1/S and G2/M (8). During G1/S the cell membrane becomes hyperpolarized relative to the resting potential and potassium channels from the voltage-gated and calcium-sensitive families respond to flux K⁺ out of the cells. In G2/M the cell membrane becomes depolarized and K⁺ flux is decreased, with a corresponding increase in CI channel conductance (9). In addition to the long recognized role of ion channels in cellular proliferation, the reverse is also true, as mitogens have been shown to up-regulate potassium channels including Kv1.3 (10, 11).

The cellular signaling pathways that regulate differentiation between TCM and TEM lymphocytes remain incompletely described. While there are strong similarities between murine and human memory cells, the voltage-gated potassium channel, Kv1.3, has been reported to have unique functions in human lymphocytes that differ in murine systems due to compensatory activation of a chloride channel in mice in which Kv1.3 was knocked out (12). We and others have previously

* This work was supported, in whole or in part, by National Institutes of Health Grant R01NS041435 (to P. A. C.).
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§ The abbreviations used are: TCM, central memory T; TEM, effector memory T; DN, dominant negative; Cdk, cyclin-dependent kinase; CaMKII, calcium-calmodulin-dependent kinase II; PBMC, peripheral blood mononuclear cells; BrdU, 5'-bromo-2'-deoxyuridine; ChIP, chromatin immunoprecipitation; SBE, SMAD binding elements.
demonstrated that T_{EM} preferentially up-regulate expression of the outward rectifying Kv1.3 channel, and that pharmacological blockade of this channel inhibits a variety of effector functions of human T cells in vitro, and in vivo rat autoimmune models including delayed type hypersensitivity and relapsing EAE (13–15). We also previously reported that long-term functional blockade of Kv1.3 in human T cells using a dominant negative (Kv1.xDN) transduction strategy not only selectively inhibited T_{EM} proliferation and cytokine production, but further caused inhibition of T_{CM} differentiation into T_{EM} (13, 16). In the present study, we sought to elucidate the mechanisms by which this channel regulates cell cycle and its role in T cell differentiation.

Our current data show that a Kv1.3-dependent signaling pathway is a critical regulator of T_{EM} cell differentiation. A loss of function mutation of Kv1.3 inhibited differentiation of T_{CM} into T_{EM} and led to conversion of T_{EM} to T_{CM}. This loss of function mutation further resulted in a concomitant delay in cell cycle at the G2/M phase. Inhibition of Kv1.3 led to enhanced translocation of phosphorylated SMAD3 to the nucleus where it binds the p21 promoter and suppresses the cell cycle-related genes cyclin-dependent kinase (Cdk)1 and cyclin B1, indicating an inhibition in cell cycle progression. These data provide a mechanism by which the pharmacological blockers may mediate their therapeutic effect and further, suggest that the signaling pathways that suppress strong T cell activation may favor T cell survival and memory.

**EXPERIMENTAL PROCEDURES**

**Isolation of CD4+ T Cells from Peripheral Blood**—Human peripheral blood mononuclear cells (PBMC) were purified from whole blood using ficoll gradients as described previously (17). CD4 subsets were obtained by negative selection using magnetic microbeads (MiltenyiBiotec, Auburn, CA). Briefly, PBMC were incubated with CD4+ T cell biotin-antibody mixture at 4 °C for 10 min, followed by 15 min of incubation with anti-biotin microbeads, and negatively separated using a MACS apparatus. The purity of human T cells was consistently >95% as routinely checked by FACS analysis.

**Flow Cytometric Analysis and Cell Sorting**—Single cell suspensions were prepared and stained as previously described (17). The monoclonal Abs utilized for the cell surface staining were FITC-anti-CD4 (PharMingen), PerCP-anti-CD4 (PharMingen), PE-anti-CCR7 (R and D systems), and APC-anti-CD45RA (PharMingen). Briefly, cells were washed twice in PBS/0.5% BSA and incubated with a mixture of Abs for 30 min on ice. Cells were washed twice again in PBS/0.5% BSA. Stained cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Immunocytometry Systems, San Jose, CA). The CD4+ cells were separated into T_{EM}, T_{CM}, and naive subsets by cell sorting using the combination of anti-CD4-Chrome, anti-CCR7-PE and anti-CD45RA-FITC mAbs. Single cell suspensions were stained, and the T_{EM}, T_{CM} and naive cells within the gate of CD4+ cell population were sorted based on their differential expression of CCR7 and CD45RA using a MoFlo MLS high-speed cell sorter (Beckman Coulter, Miami, FL). The purity of each sorted population was consistently >95%.

**RT-PCR**—RNA was isolated using RNeasy mini kit from Qia-gen for total RNA purification. cDNA was made using Super-Script® III First-Strand Synthesis System for microarray analysis.

**Gene Microarray Analysis**—The RNA samples were analyzed with Affymetrix-GeneChiphuman 133 2.0 Arrays. Quality of the microarray experiment was assessed with AffyPLM and Affy, two bioconductor packages for statistical analysis of microarray data. To estimate the gene expression signals, data analysis was conducted on the chips’ CEL file probe signal values at the Affymetrix probe pair (perfect match (PM) probe and mismatch (MM) probe) level, using the statistical algorithm Robust Multi-array expression measure with Affy. This probe level data processing includes a normalization procedure utilizing quantile normalization method to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization, and/or scanning.

**Exploratory Data Analysis (EDA)**—EDA was performed with the normalized data. Multi-Dimensional Scaling (MDS) was performed with R function isoMDS to assess the closeness among samples. Between-condition and between-replicate variation was examined with pairwise Mva plots, in which the base 2 log ratios (M) between two samples are plotted against their averaged base 2 log signals (A). With the signal intensities estimated above, an empirical Bayes method with the Gamma-Gamma modeling, as implemented in the bioconductor package EBarrays, was used to estimate the posterior probabilities of the differential expression of genes between the GFP-control and GFP-Kv1.3DN (18). The criterion of the posterior probability >0.5, that is to say the posterior odds favors change, was used to produce the differentially expressed gene lists.

**Cell Cycle Assay**—A 5'-bromo-2'–deoxyuridine (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 (1 × 10^6 per well) were cultured with 10 μM BrdU, and incubations continued for an additional 4 h. Cells were fixed in a solution containing paraformaldehyde and the detergent saponin, and incubated for 1 h with DNase at 37 °C (30 μg per sample). APC-conjugated anti-BrdU antibody (1:50 dilution in Wash buffer; BD Pharmingen, San Diego, CA) was added and incubation continued for 20 min.
at room temperature. Cells were washed in Wash buffer and total DNA was stained with 7-amino-actinomycin D (7-AAD; 20 μg/ml per sample). BrdU content (APC) and total DNA content (7-AAD) were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

PKH26 Labeling of Transduced CD4+ T Cells—Transduced CD4+ T cells (1 × 10⁷) were labeled with PKH26 dye solution according to the manufacturers’ recommendations (Sigma-Aldrich).

Immunofluorescence Staining—Cells were washed and placed into cytopsin funnels and spun onto glass slides using

FIGURE 1. Lentiviral transduction with a dominant-negative Kv1.x construct inhibits T_EM cells. A, purified CD4+ T cells were stained with fluorescent conjugated anti-CD4, anti-CCR7 and anti-CD45RA mAbs. Subsequently, T EM, T CM, and naive T cell subpopulations were sorted from the CD4+ gated cell population. B, sorted T EM, T CM, and naive T cells within the respective gates shown were stimulated with anti-CD3/CD28 for 24 h and then transduced with a lentiviral vector encoding the DNKv1.x-GFP or GFP control alone at an MOI of ~5. After 7 days of transduction, cells were stained with anti-CD4, anti-CCR7 or anti-CD45RA and analyzed for the percentages of T EM, T CM and naive cells within the gated GFP+ CD4+ cells. The gate for expression of GFP was established using untransduced controls. The plots shown are representative data from three separate experiments using cells from different donors. C, percentages of each CD4+ subset displaying GFP fluorescence are presented as mean ± S.D. of three experiments. Values that are significantly different from that of GFP control cells are indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.005. D, FACS sorted CCR7+ TEM CD4 cells were labeled with PKH26 (2 μg/ml), followed by stimulation with anti-CD3/CD28 for 24 h and then transduced with a lentiviral vector encoding the DN-Kv1.x and GFP control alone at an MOI of ~5. CCR7 expression was measured in GFP and DN-Kv1.x-transduced TEM CD4 T cells labeled with PKH26 at the indicated timepoints. The data are representative of two experiments. E, purified CD4+ T cells were stimulated with soluble anti-CD3 (1 μg/ml), anti-CD28 Abs (1 μg/ml), irradiated PBMC, and recombinant human IL-2 (20 units/ml). The culture was maintained by biweekly restimulation with the above stimuli for 6 weeks. Under this in vitro repeated antigen stimulation, >90% of cells were terminally differentiated T EM cells (day 0). In vitro generated chronic TEM cells were then subjected to transduction with DN-Kv1.x and GFP control alone at an MOI of ~5. After 12 and 19 days of transduction, cells were stained with anti-CD4 and anti-CCR7. F, percentages of CCR7+ GFP+ cells are presented as mean of triplicate ± S.D. of one representative of two experiments. The value was significantly different from that of GFP control. (**, p < 0.01; *** p < 0.005).
a cytospin centrifuge (Shandon, Pittsburgh, PA) and subsequently fixed with 3.7% paraformaldehyde, washed, and blocked. Thereafter, cells were incubated with rabbit anti-human SMAD3 or phospho-SMAD3 (Ser-423/425) (Alamone Labs, Jerusalem, Israel) antibodies for 30 min at room temperature. Cells were thereafter labeled with donkey anti-rabbit IgG secondary antibodies conjugated to Alexa Fluor (AF)-594 (Molecular Probes, Eugene, OR). Cellular nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) at 1 μg/ml for 10 min. After being mounted in ImmunoFluore medium (ICN Biomedicals, Aurora, OH), images were acquired by OpenLab software on a Zeiss Axiovert S100 microscope under ×100 objective (Carl Zeiss, Thornwood, NY).

Western Blotting—Nuclear and cytoplasmic extracts were prepared from DNKv or GFP control cells using the CelLyticNuCLEAR extraction kit from Sigma according to the manufacturer’s instructions. Phosphatase inhibitors were also added to the lysate. Protein was quantified using the BCA assay (Pierce) and 30 μg of lysate was used for SDS-PAGE. Western blots were performed using antibodies specific for p21, p27 (Millipore, Temecula, CA), cyclin B1, Cdk1, pSMAD3, SMAD3 (Cell Signaling Technology, Danvers, MA), and actin (Sigma). Blots were initially probed for p21 or pSMAD3 and stripped and reprobed for additional proteins. Average densitometric ratio was calculated for three replicate experiments using Adobe Photoshop software and graphed as percent of maximum average densitometric ratio.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assay was conducted as previously described (19). Briefly, DNKv or GFP control transduced T cells were restimulated with anti-CD3 and anti-CD28 for 6 h and then transduced with a lentiviral vector encoding the DN-Kv1.x and GFP control alone at an MOI of ~5. After 7 days of transduction, cultures were pulsed with 10 μM BrdU for the final 18 h. Cell cycle was analyzed by examining incorporated BrdU and total DNA levels (7AAD) by flow cytometry. Non-infected T-cells as well as cells containing no BrdU were used as negative control. The plots shown are representative data from three separate experiments using cells from different donors. The percent GFP− cells in each phase from transduced CD4+ cells (B) and TEM subpopulation (D) are expressed relative to GFP control cells (control data were set to 100%). The data are expressed as mean ± S.D. of three experiments. Values that are significantly different from that of GFP control cells are indicated as *, p < 0.05; **, p < 0.01; ***, p < 0.005.
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**RESULTS**

**Kv1.3 Loss of Function Mutation Intrinsically Interferes with T<sub>EM</sub> Differentiation**—To investigate novel targets of Kv1.3 blockade in T lymphocytes, we first assessed changes in gene expression profiles from human T cells in which Kv1.3 function was inhibited with a KvDN using an AffymetrixGeneChips human 133 2.0 array. Gene family cluster analyses revealed notable changes in ion channels, cell cycle genes, and in T cell regulation and cellular differentiation pathways including TGFβ signaling pathway members (supplemental Table S1). Because Kv1.3 is predominantly expressed in T<sub>EM</sub>, we sought to determine the mechanism by which accumulation of T<sub>CM</sub> occurred in our prior report. We therefore explored the possibility that Kv1.3 might alter the plasticity of already established T<sub>EM</sub>. We first sorted primary human CD4<sup>+</sup> T cells into T<sub>CM</sub>, T<sub>EM</sub>, and naïve subsets based on the expression of CCR7 and CD45RA (Fig. 1A), and transduced each type with KvDN or GFP control lentiviral vectors. As expected, after stimulation for 7 days, GFP control transduced cells from the T<sub>CM</sub> subset differentiated into T<sub>EM</sub>, whereas KvDN-transduced cells failed to differentiate and remained predominantly T<sub>CM</sub> (Fig. 1B and C). KvDN cells demonstrated a significantly greater increase in CCR7 up-regulation and reversion to a CCR7<sup>+</sup> phenotype compared with the controls. To ascertain whether the observed accumulation of T<sub>CM</sub> was indeed derived from T<sub>EM</sub> and not a small contaminating pool of T<sub>CM</sub> we labeled the T<sub>EM</sub> cells with the membrane marker PKH and analyzed their coordinated levels of CCR7 expression and cellular division (Fig. 1D). Consistent with the notion of T<sub>EM</sub> plasticity, significantly more of the labeled KvDN-transduced T<sub>EM</sub> reverted to CCR7<sup>+</sup> T<sub>CM</sub> and exhibited slowed proliferation as compared with the control T<sub>EM</sub> cells. This reversal was evident in both primary isolated T<sub>EM</sub> cells and chronically activated (in vitro) T<sub>EM</sub> (Fig. 1E and F), as well as in cells stimulated with anti-CD3 alone (supplemental Fig. S5), consistent with an effect on co-stimulation-independent effector memory T cells. Cell viability was equal in both control and KvDN pools as measured by Annexin V staining (supplemental Fig. S1).

**Kv1.3 Loss of Function Mutation Causes a Delay of Cell Cycle in G2/M Phase in T<sub>EM</sub> Cells**—To assess the relationship between cell cycle progression and the diminished capacity of Kv-blocked T<sub>EM</sub> cells to proliferate and differentiate, we performed a cell cycle analysis of control and KvDN-transduced T cells. As shown in Fig. 2, A and B, CD4<sup>+</sup> T cells transduced with KvDN display a significantly greater proportion of cells in G2/M phase when compared with control cells. We fractionated the cells into distinct subsets and observed no significant difference in control and KvDN cells in cell cycle profiles in T<sub>CM</sub> and naïve subsets. In contrast, T<sub>EM</sub> cells transduced with KvDN had significant increases in the numbers of cells in G2/M phase, relative to GFP control T<sub>EM</sub> cells (Fig. 2, C and D). Treatment of T cells with the DNA synthesis inhibitor, aphidicolin, inhibited proliferation and caused S phase arrest but did not increase levels of CCR7 (supplemental Fig. S2).

**Kv1.3 Loss of Function Mutation Enhances SMAD3 Expression in the Nucleus**—To investigate novel targets of Kv1.3 loss of function, we transfected T cells with a dominant-negative Kv1.x construct increased p21 and impaired Cdk1 and cyclin B1 expression in the nucleus. FACs-sorted GFP<sup>+</sup> cells from transduced CD4 T cells at day12 were rested for 24 h and then stimulated with anti-CD3/CD28 for 6 and 24 h. A, cytoplasmic and nuclear protein extracts were analyzed for p21, cyclin B1, and Cdk1 by Western blot. B, quantification of p21, p27, cyclin B1, and Cdk1 expressions for both nuclear and cytoplasmatic fractions relative to β-actin. Experiments were carried out in triplicate, and the data are presented as values normalized against β-actin protein.
that calmodulin regulates SMAD signaling, we measured the expression and phosphorylation of SMAD3 in control and KvDN transduced T cells (20). Our results indicate that a loss of function mutation of Kv1.3 led to an increase in expression of pSMAD3 as compared with GFP control cells (Fig. 4, A–C). Further, Western blot analysis demonstrated a significant accumulation of pSMAD3 in the nucleus of KvDN cells when compared with GFP control cells (Fig. 4, D and E). An increase in phosphorylated SMAD3 was also seen when CD4+ T cells were stimulated in the presence of the pharmacological Kv1.3 blocker, margatoxin, and was equivalent to the change seen during canonical TGF-β-induced signal transduction (supplemental Fig. S3). These data suggest that Kv1.3 blockade, achieved either with the use of a pharmacological inhibitor or the dominant negative Kv1.x construct, enhances SMAD3 phosphorylation and translocation of pSMAD3 into the nucleus where it regulates transcription of target genes.

**Transcriptional Regulation of p21 Expression by SMAD3 in KvDN Cells**

SMAD3 is a canonical TGF-β/activin-induced transcription factor with a variety of downstream effects on transcription. Regulation of p21 expression by SMAD3 in KvDN cells was investigated. Purified CD4+ T cells were stimulated with anti-CD3/CD28 for 24 h. Subsequently, activated CD4+ T cells were transduced with a lentiviral vector encoding DN-Kv1.x and a control vector at an MOI of 5. After 10 days of transduction, (A) cells were serum starved for 48 h and then restimulated with anti-CD3 or anti-CD3/CD28 for 72 h. Cells were stained with SMAD3 and phospho-SMAD3 (Ser-423/425)-specific mAbs for flow cytometric analysis on GFP+ CD4+ cells from GFP control (gray line) and KvDN (black line). The plots shown are representative data from three separate experiments. B, magnitude of SMAD3 and phospho (serine 423–5)-SMAD3 expression was defined by the mean fluorescence intensity (MFI). Data are presented as mean ± S.D. of three experiments. Values that are significantly different from that of GFP control cells are indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.005. C, GFP+ cells were sorted from both GFP and DN Kv1.x-transfected CD4+ cells by FACS, followed by 48 h serum starvation. Cells were then restimulated with anti-CD3/CD28 for 72 h. Cells were immunostained for SMAD3 (red) and phospho-SMAD3 (Ser-423/425) (red) and subsequently viewed by immunofluorescence microscopy. Cellular nuclei were counterstained with DNA dye DAPI (blue). An isotype-matched antibody was used as a negative control. Original magnification, ×100. D, FACS-sorted GFP+ cells from transduced CD4+ T cells at day12 were rested for 24 h and then stimulated with anti-CD3/CD28 for 6 and 24 h. Cytoplasmic and nuclear protein extracts were analyzed for SMAD3 and phospho-SMAD3 by Western blot. E, protein expression was quantified according to average densitometric ratio. Experiments were performed in triplicate, normalized to actin, and presented as percent of maximum average densitometric ratio.
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**A**

- **p21 promoter**
  - forward arrow to -1752 to -1733 site
  - reverse arrow from -1752 to -1733

**B**

- **DNKv**
  - Input α-SMAD3 α-IgG
- **GFP Ctrl**
  - Input α-SMAD3 α-IgG

**C**

- **α-SMAD3**
- **α-IgG**

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**FIGURE 5. Regulation of p21 via SMAD3 in DN-Kv1.x-transduced cells.** A, schematic illustration of the p21 promoter depicting the location of SBE relative to the transcription start site. B, FACS-sorted GFP<sup>+</sup> cells from transduced CD4<sup>+</sup> T cells at day12 were rested for 24 h and then stimulated with anti-CD3/CD28 for 6 h. ChIP was performed with anti-SMAD3 antibody or IgG control antibody. Quantitative PCR was performed using primers amplifying the p21 promoter from -1752 to -1733 bp. PCR product was run on a gel and binding for DN-Kv transduced cells was compared with GFP controls. Lane 1, input; lane 2, immunoprecipitated with anti-SMAD3; lane 3, immunoprecipitated with IgG control. C, quantitative PCR results are graphed as percent of input. A representative result from at least three independent experiments is shown. The data are expressed as mean ± S.D. of three experiments. Values that are significantly different from that of GFP control or IgG control are indicated as *, p < 0.05; **, p < 0.01; ***, p < 0.005.

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**DISCUSSION**

We report that a loss of function mutation of Kv1.3 resulted in suppression of CD4<sup>+</sup> T cell differentiation into T<sub>EM</sub>, and further, enhanced reversion of T<sub>EM</sub> to T<sub>CM</sub>. Results of our mechanistic studies suggest that the effect is related to a delay in cell cycle at G2/M and induction of SMAD3-mediated expression of p21<sup>cip1</sup>, but not p27<sup>kip1</sup>, with resulting suppression of Cdk1 and cyclin B1. These data have several implications for elucidating the process of T cell lineage differentiation and provide new evidence for the importance of the role of ion channels in regulating not only cell cycle, but also the immunologic state and function of T cells. Because T<sub>EM</sub> have been identified in the target organs of several autoimmune diseases, understanding the signaling pathways that lead to expansion and reversion of memory cells may allow for the development of specific targeted therapies for T<sub>EM</sub> cells in these diseases.

The maintenance of immunological memory within the T<sub>CM</sub> pool has been attributed to either asymmetric cell division or reversion of differentiated T<sub>EM</sub> to T<sub>CM</sub>, but the signaling mechanisms that underlie T cell memory lineage fate decisions have not been well delineated. While several specific transcription factors have been shown to be necessary for polarization of T cells toward specific cytokine profiles, and SMAD3 is known to be important in TGFβ and T cell regulation, the role of SMAD3 in T<sub>EM</sub> to T<sub>CM</sub> differentiation has not been explored. Kv1.3 blockade results in calcium depletion and SMAD3 phosphorylation, which may lead to the induction of the lymph node homing receptors CCR7 and CXCR4.

As activation of T cells is associated with cell division and progression through the cell cycle, we hypothesized that a loss of function mutation of Kv1.3 might be affecting differentiation via an effect on cell cycle progression. Regulation of cell cycle progression occurs through coordinated expression and suppression of numerous Cdk's by members of the cyclin-dependent kinase inhibitor protein (cip/kip) family. Two members of the cip/kip family of cyclin-dependent kinase inhibitors, p21<sup>cip1</sup> and p27<sup>kip1</sup>, have been shown to play important roles in T cell anergy. p27<sup>kip1</sup> functions to maintain cells in G1 until appropriate stimulation occurs, and is suppressed by costimulatory signals such as CD28 and IL-2 (22–24). p21<sup>cip1</sup> likely plays a complementary role to p27<sup>kip1</sup> by regulating cell cycle inhibitors Cdk1 and cyclin B1 at G2/M, as shown herein under conditions of strong costimulation, which should repress p27<sup>kip1</sup>. Thus, the prior observation that G1/S arrest by itself does not restore antigen responsiveness, but SMAD3 knockdown mutant does, indicates a critical role for SMAD3 signaling in anergy, independent of p27<sup>kip1</sup> (24, 25).

TGFβ/SMAD signaling is regulated upstream by levels of intracytoplasmic calcium and the calcium-calmodulin-dependent kinase II (CaMKII), which prevents SMAD3 from complexing with SMAD2 and translocating to the nucleus (26). Thus, when intracellular calcium levels are depleted, as occurs during Kv1.3 blockade, SMAD3 should more easily complex with SMAD2 and undergo phosphorylation at the C-terminal serine 423–5 site, which is necessary for transcriptional activation of downstream factors (27), such as p21 (Fig. 6).

Interestingly, lymphocytes express Kv1.3 channels both at their plasma membrane and in organelles, such as mitochondria. The studies performed herein utilized a loss of function mutation that is expected to affect channel expression at both locations, as well as the pharmacological Kv1.3 inhibitor margatoxin, which is a non-cell permeable inhibitor that would be
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expected to affect only channels expressed at the plasma membrane. The similarity of results seen with both methods of functional blockade suggest a role for Kv1.3 channels at the plasma membrane, but not for those expressed in organelles, in regulating differentiation via an effect on cell cycle progression and SMAD3 phosphorylation.

In summary, our findings support a novel role for ion channels in regulating T cell differentiation. Remarkably, in cells with a Kv1.3 loss of function mutation, not only do T_{CM} fail to differentiate into T_{Em}, but enhanced reversion of T_{Em} into T_{CM} was observed. This effect was traced to enhanced SMAD3 signaling and subsequent induction of p21 and suppression of cyclin B1 and Cdk1 linking this pathway with the observed effects. These findings are consistent with the hypothesis that the strength of T cell signal may determine T_{CM} to T_{Em} differentiation, but suggests this process is more reversible than previously thought and that T lymphocyte plasticity may directly relate to calcium modulation by ion channels.

Acknowledgment—We thank Connie Talbot at the Johns Hopkins Gene Array Core Facility.

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FIGURE 6. A model for Kv1.3-mediated signaling pathway in human T effector memory cell differentiation. Kv1.3 blockade by KvDN depletes the cytoplasmic Ca^{2+} that inhibits the activation of the CaMII. The down-regulation of CaMII signaling capable of preventing SMAD3 from complexing with SMAD2 leads to increased recruitment of SMAD3 into the SMAD transcriptional complex and translocation to the nucleus, which in turn results in increased p21 expression. The overexpression of p21 reduces the complex formation between Cdk1 and cyclin B1, thereby causing G2/M phase cell cycle arrest.

A model for Kv1.3-mediated signaling pathway in human T effector memory cell differentiation. Kv1.3 blockade by KvDN depletes the cytoplasmic Ca^{2+} that inhibits the activation of the CaMII. The down-regulation of CaMII signaling capable of preventing SMAD3 from complexing with SMAD2 leads to increased recruitment of SMAD3 into the SMAD transcriptional complex and translocation to the nucleus, which in turn results in increased p21 expression. The overexpression of p21 reduces the complex formation between Cdk1 and cyclin B1, thereby causing G2/M phase cell cycle arrest.

expected to affect only channels expressed at the plasma membrane. The similarity of results seen with both methods of functional blockade suggest a role for Kv1.3 channels at the plasma membrane, but not for those expressed in organelles, in regulating differentiation via an effect on cell cycle progression and SMAD3 phosphorylation.

In summary, our findings support a novel role for ion channels in regulating T cell differentiation. Remarkably, in cells with a Kv1.3 loss of function mutation, not only do T_{CM} fail to differentiate into T_{Em}, but enhanced reversion of T_{Em} into T_{CM} was observed. This effect was traced to enhanced SMAD3 signaling and subsequent induction of p21 and suppression of cyclin B1 and Cdk1 linking this pathway with the observed effects. These findings are consistent with the hypothesis that the strength of T cell signal may determine T_{CM} to T_{Em} differentiation, but suggests this process is more reversible than previously thought and that T lymphocyte plasticity may directly relate to calcium modulation by ion channels.

Acknowledgment—We thank Connie Talbot at the Johns Hopkins Gene Array Core Facility.

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FIGURE 6. A model for Kv1.3-mediated signaling pathway in human T effector memory cell differentiation. Kv1.3 blockade by KvDN depletes the cytoplasmic Ca^{2+} that inhibits the activation of the CaMII. The down-regulation of CaMII signaling capable of preventing SMAD3 from complexing with SMAD2 leads to increased recruitment of SMAD3 into the SMAD transcriptional complex and translocation to the nucleus, which in turn results in increased p21 expression. The overexpression of p21 reduces the complex formation between Cdk1 and cyclin B1, thereby causing G2/M phase cell cycle arrest.