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Characterization of the Functional Properties of the Voltage-Gated Potassium Channel Kv1.3 in Human CD4+ T Lymphocytes

Lina Hu,* Michael Pennington,† Qiong Jiang,‡ Katharine A. Whartenby,† and Peter A. Calabresi2*

Previous studies have shown that central memory T (TCM) cells predominantly use the calcium-dependent potassium channel KCa3.1 during acute activation, whereas effector memory T (TEM) cells use the voltage-gated potassium channel Kv1.3. Because Kv1.3-specific pharmacological blockade selectively inhibited anti-CD3-mediated proliferation, whereas naive T cells and TCM cells escaped inhibition due to up-regulation of KCa3.1, this difference indicated a potential for selective targeting of the TEM population. We examined the effects of pharmacological Kv1.3 blockers and a dominant-negative Kv1.x construct on T cell subsets to assess the specific effects of Kv1.3 blockade. Our studies indicated both TCM and TEM CD4+ T cells stimulated with anti-CD3 were inhibited by charybdotoxin, which can block both KCa3.1 and Kv1.3, whereas margatoxin and Stichodactyla helianthus toxin, which are more selective Kv1.3 inhibitors, inhibited proliferation and IFN-γ production only in the TEM subset. The addition of anti-CD28 enhanced proliferation of freshly isolated cells and rendered them refractory to S. helianthus, whereas chronically activated TEM cell lines appeared to be costimulation independent because Kv1.3 blockers effectively inhibited proliferation and IFN-γ regardless of second signal. Transduction of CD4+ T cells with dominant-negative Kv1.x led to a higher expression of CCR7+ TCM phenotype and a corresponding depletion of TEM. These data provide further support for Kv1.3 as a selective target of chronically activated TEM without compromising naive or TCM immune functions. Specific Kv1.3 blockers may be beneficial in autoimmune diseases such as multiple sclerosis in which TEM are found in the target organ. The Journal of Immunology, 2007, 179: 4563–4570.

The voltage-gated potassium channel, Kv1.3, was first identified on T lymphocytes over two decades ago. In T lymphocytes, Kv1.3 subunits form homotetrameric complexes, which markedly increases this channel’s expression relative to other cell types in which the Kv1.3 subunits are associated with other Kv1-family subunits to form functional heteromultimeric channels. Kv1.3 and the Ca2+-dependent potassium channel KCa3.1 (also known as IKCa1) maintain the membrane potential by promoting a countercurrent efflux of potassium to allow influx of extracellular calcium through calcium-release-activated channels (1–3). This process is critical for maintenance of T cell activation because intracellular stores of calcium are rapidly depleted.

Our recent findings indicate that Kv1.3 and KCa3.1 are differentially expressed on three human CD4+ T cell subsets, naive (CCR7+CD45RA+), central memory T (TCM, CCR7+CD45RA+), and effector memory T (TEM, CCR7−CD45RA−) cells, depending on the state of cell activation (1, 4). In quiescent naive, TCM, and TEM T cells, Kv1.3 channels (250–400 channels/cell) predominate (4, 5). KCa3.1 channels are up-regulated in naive and TEM cells after activation (increasing from 10 to 500/cell), and become the predominant functional K+ channel in these cells during activation (4–6). In contrast, Kv1.3 is up-regulated to 1500–2000 channels/cell in activated TEM cells such that the TEM subset expresses 6-fold higher numbers of Kv1.3 channels in their membranes, but low levels of KCa3.1 channels (4, 7).

The relevance of Kv1.3 to disease has been demonstrated by several animal and human studies, and its potential for a target to treat disease has been investigated. In one set of experiments, Kv1.3-specific pharmacological blockers improved the course of disease in experimental autoimmune encephalomyelitis if either the myelin basic protein (MBP)-reactive T cells used to induce disease were treated in vitro or if the rats themselves were treated in vivo after T cell transfer (8, 9). In addition, we have shown that MBP-reactive T cells from patients with multiple sclerosis (MS) had a Kv1.3high/KCa3.1low phenotype relative to control MBP-reactive T cells and nondisease-related Ags (4). Furthermore, Kv1.3 expression is seen upon activation of infiltrating cerebrospinal fluid cells and is high on parenchymal T cells in MS brain tissue (10). Thus, the activated autoreactive TEM T cells, which express the Kv1.3high/KCa3.1low phenotype, may play an important pathogenic role in MS, presumably by homing specifically to the CNS, where they exhibit immediate effector functions resulting in the recruitment and activation of macrophages and microglia and

sclerosis; ShK, Stichodactyla helianthus toxin; TCL, T cell line; TEM, effector memory T.

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Abbreviations used in this paper: TCM, central memory T; ChTx, charybdotoxin; DN, dominant negative; MBP, myelin basic protein; MgTx, margatoxin; MS, multiple sclerosis; ShK, Stichodactyla helianthus toxin; TCL, T cell line; TEM, effector memory T.

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perhaps by mediating direct damage to oligodendrocytes, neurons, and axons (11, 12).

Although Kv1.3 has been shown to play a critical role in T cell activation and proliferation, previous studies have not examined the potential for different effects of Kv1.3 blockers on T cell subsets. In the present study, we used both pharmacological Kv1.3 blockade and a dominant-negative (DN) Kv1.x construct to explore the effects of Kv1.3 inhibition on proliferation of naive, TCM, and TEM cell subsets. Our data showing a preferential inhibitory effect of these blockers on TEM provide further evidence that Kv1.3 channel expression on costimulation-independent TEM could potentially be a selective therapeutic target for this subset of T cells, which has been implicated in autoimmune diseases.

Materials and Methods
Isolation of CD4⁺ T cells from peripheral blood
Human PBMC were purified from whole blood using Ficoll gradients, as described previously (13). CD4 subsets were obtained by negative selection using magnetic microbeads (Miltenyi Biotec). Briefly, PBMC were incubated with CD4⁺ T cell biotin-Ab mixture at 4°C for 10 min, followed by 15-min 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.

Generation of terminally differentiated T EM cells and myelin-reactive T cell lines (TCLs) in vitro
Freshly isolated human CD4⁺ T cells were adjusted to 1 × 10⁶/ml in T cell medium (IMDM) supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml) (Bio-Whittaker), and 5% human serum (Sigma-Aldrich)). The cells were stimulated with soluble anti-CD3 (1 μg/ml), anti-CD28 Abs (1 μg/ml), irradiated (3000 rad) PBMC, and human rIL-2 (Biological Resources Branch, National Institutes of Health), and maintained by biweekly re-stimulation with the above stimuli for 6 –10 wk. The percentage of TEM cells within the gate of CD4⁺ cell population was assayed using the combination of anti-CD4 FITC, anti-CCR7 PE, and anti-CD45RA allophycocyanin (BD Pharmingen). This procedure yielded a cell population containing >90% T EM cells. TCLs specific for MBP (Sigma-Aldrich) were generated using PBMC derived from three separate MS patients, as previously described (4).

T cell activation
Freshly isolated CD4⁺ T cells and in vitro generated T EM cells were re-suspended at 1–2 × 10⁶ cells/ml in complete IMDM medium, mixed with anti-CD3 alone (cells:beads, 1:1) or anti-CD3/CD28 (cells:beads, 10:1) mAb-conjugated magnetic beads (Dynal Biotech), and incubated for 4–5 days.

Channel blockers
Three K⁺ channel blockers were used in the functional assays, including the following: Stichodactyla helianthus toxin (ShK), a potent Kv1.3 inhibitor (2); margatoxin (MgTx), a specific Kv1.3 inhibitor (14, 15); and charybdotoxin (ChTx), which inhibits both Kv1.3 (16 –18) and KCa3.1 (19, 20) channels with similar potency. ChTx and MgTx were purchased from Alomone Laboratories. ShK was provided by M. Pennington (Bachem, King of Prussia, PA). Concentrations of blockers were chosen based on inhibition of T cell proliferation in preliminary assays using the cell:bead ratios stated above.

Flow cytometric analysis and cell sorting
Single-cell suspensions were prepared and stained, as previously described (13). The mAbs used for the cell surface staining were anti-CD4 FITC (BD Pharmingen), anti-CD4 PerCP (BD Pharmingen), anti-CCR7...
PE (BD Pharmingen), and anti-CD45RA allophycocyanin (BD 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 FACSCalibur flow cytometer using CellQuest software (BD Immunocytometry Systems).

The CD4⁺/H11001 cells were separated into TEM, T CM, and naive T cell subpopulations by cell sorting using the combination of anti-CD4 CyChrome, anti-CCR7 PE, and anti-CD45RA FITC mAbs. Single-cell suspensions were stained, and the TEM, T CM, and naive T cells within the respective gates were sorted based on their differential expression of CCR7 and CD45RA using a MoFlo MLS high-speed cell sorter (DakoCytomation). The purity of each sorted population was consistently >95%.

[3H]Thymidine incorporation
Freshly isolated CD4⁺ T cells and in vitro generated TEM cells were pretreated with channel blockers for 3 h. The cells were then cultured at 2 × 10⁶ cells/ml in triplicate in 96-well flat-bottom plates (Falcon) in 0.2 ml of IMDM/5% human serum/1× penicillin-streptomycin under the stimulation conditions, as described above, for 4–5 days. Cultures were pulsed with 1 μCi of [³H]thymidine (Amersham Biosciences) for the final 18 h. Cells were harvested by a 96-well plate harvester (PerkinElmer), and [³H]thymidine uptake was measured in a liquid scintillation counter (PerkinElmer).

CFSE staining
In vitro generated TEM cells were suspended in PBS containing 1% FCS at a concentration of 10⁷ cells/ml and labeled with the fluorochrome CFSE (Molecular Probes). Cells were incubated at 37°C for 10 min with CFSE at a final concentration of 1 μM, washed twice with, and then resuspended in IMDM/5% human serum/1× penicillin-streptomycin. The dilution of CFSE was measured by flow cytometric analysis after 5 days of culture under the stimulation conditions as described above.

ELISA analysis
 Supernatants of T cell cultures were collected and stored at –80°C. The frozen supernatants were thawed at room temperature, and cytokine levels were measured by assays using commercial kits for IFN-γ and IL-5 (BioSource International), according to the manufacturers’ instructions.
Lentiviral transduction of activated CD4⁺ T cells

The lentiviral vector particles were produced by transient transfection of three plasmids, as follows: the vesicular stomatitis virus G protein envelope encoding construct pMD.G, the packaging construct pCMVΔ8.91, and vector plasmid that contains the GFP gene and DN Kv1.x sequence, which codes for a Kv1.x molecule with a function-blocking mutation (GYG to AYA) in the pore-forming region (21, 22) into 293T cells. The DN protein prevents formation of the tetrameric complex in the membrane and thus blocks the functional channel pore. It is specific for Kv1 family proteins, but because human T cells are only known to express Kv1.3, this channel is likely to be the only one affected.

To obtain activated CD4⁺ T cells, highly purified CD4⁺ T cells were stimulated with anti-CD3/CD28 for 24 h, and then transduced with a lentiviral vector encoding the DN Kv1.x and GFP or control (CTL) GFP alone at a multiplicity of infection of ~3. Cells were stained with anti-CD4, anti-CCR7, or anti-CD45RA mAbs at the indicated time points and analyzed for the percentages of TEM, TCM, and naive cells within the gated GFP⁺ CD4⁺ cells. The gate for expression of GFP was established using untransduced controls. A, The plots shown are representative data from five separate experiments using cells from different donors. The percentage of cells in each quadrant is indicated. B, The percentages of each CD4⁺ subset displaying GFP fluorescence are presented as mean ± SD of all five experiments. Values that are significantly different from that of GFP control virus-infected cells are indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.005.

Results

Kv1.3 channel blockers do not inhibit activation of freshly isolated CD4⁺ T cells

We have previously shown that blockers of Kv1.3 channels are able to inhibit anti-CD3-mediated T cell proliferation (4). To assess the effect of Kv1.3 channel blockade on cell proliferation when a second signal was present, we stimulated freshly isolated CD4⁺ T cells with either anti-CD3 alone or in combination with anti-CD28 (Fig. 1). Fig. 1A demonstrates that both ChTx (KCa3.1 and Kv1.3 blocker) and MgTx (Kv1.3-specific blocker) inhibited anti-CD3/CD28-mediated cell proliferation, whereas ShK (Kv1.3-specific blocker) had no significant inhibitory effect on freshly isolated CD4⁺ T cells. ShK did somewhat suppress cell proliferation mediated by treatment with anti-CD3 alone (Fig. 1B). These inhibitory effects were not due to nonspecific cytotoxicity by K⁺ channel inhibitors, because >95% T cells were viable at the time of assay as judged by both trypan uptake and ethidium monoazide staining.

We next sought to measure the effects of Kv1.3 blockade on cytokine production. Previous reports suggest that although both TCM and TEM respond to antigenic stimulation, cytokine production is greater in TEM than in TCM (23). Treatment of T cells with MgTx and ChTx significantly suppressed the secretion of both
FIGURE 4. Effect of K channel blockers on chronic TEM cells and myelin-specific TCLs. A, FACS plot showing the transition of freshly purified CD4⁺ T cells into TEM cells after repeated stimulation with anti-CD3/CD28 for 5–8 wk. B, Chronically stimulated TEM cells labeled with CFSE were pretreated with three different K channel blockers and examined by FACS after stimulation with anti-CD3/CD28 or anti-CD3 alone for 5 days. C and D, The percentages of cells proliferating was reduced by all the blockers with both C and D without costimulation. E, The levels of IFN-γ were measured in anti-CD3/CD28-stimulated T cell supernatants by ELISA and were reduced by K channel blockers. Data are mean ± SD of three different experiments. F, Kv1.3 channel blocker, ShK, inhibits the proliferation of MBP-specific TCLs derived from MS patients. The ³H incorporation by MBP-reactive TCLs treated with ShK at the indicated concentrations is shown as percentage relative to that without ShK treatment. Data are merged from proliferation assays on three different MBP-reactive TCLs. Values that are significantly different from that of nonblocker-treated controls are indicated as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.005.
IFN-γ and IL-5 cytokines. Interestingly, whereas no inhibitory effect of ShK was observed on cell proliferation in response to stimulation with anti-CD3/CD28 (Fig. 1, A and B), ShK strongly suppressed anti-CD3/CD28-stimulated IFN-γ (Fig. 1C) and IL-5 (Fig. 1D) production, which is again consistent with an effect of ShK on the effector subset.

**Kv1.3 blockers selectively suppress the proliferation of and cytokine production by T_{EM} cells**

To further identify the target subpopulation of T cells inhibited by K⁺ channel blockers, two approaches were used. First, we separated T cells into T_{EM}, T_{CM}, and naive subtypes by sorting from purified ex vivo CD4⁺ T cells, based on their surface expression of CCR7 and CD45RA (Fig. 2A), and analyzed the effects of K⁺ channel blockers on anti-CD3- or anti-CD3/CD28-stimulated cell proliferation (Fig. 2, B–E) and cytokine production (Fig. 2F). Of note, ShK significantly suppressed the proliferation of T_{EM} cells when they were stimulated with anti-CD3, but not when they were stimulated with both anti-CD3 and anti-CD28 (Fig. 2B). As expected, ShK at active concentrations on T_{EM} cells did not block the proliferation of T_{CM} cells (Fig. 2, C and E) and naive cells (Fig. 2D). MgTx was similarly effective in inhibiting anti-CD3-stimulated T_{EM} cell proliferation (Fig. 2B). The antiproliferative effects of ChTx were observed in all subsets, including T_{EM} (Fig. 2, B–D). Because it has been shown that low serum concentrations favor the suppressive activity of K⁺ channel blockers (7, 17, 24), we compared the influence of serum at lower (1%) and higher (5%) concentration on inhibitory effects of Kv1.3 channel blockers on T_{EM} cells in response to anti-CD3. ShK suppressed anti-CD3-stimulated proliferation of T_{EM} cells cultured in medium containing 1% serum more efficiently than that in 5% serum medium (Fig. 2E). Similar to its effect on T_{EM} cell proliferation, ShK significantly inhibited IFN-γ and this suppression occurred at 10 nM in 5% serum, and at concentrations as low as 1 nM ShK in 1% serum (Fig. 2F).

To more definitively assess the effects of channel blockade, we transduced CD4⁺ T cells with a GFP-tagged, lentiviral vector expressing a DN Kv1.1 sequence, which codes for a Kv1.1 molecule with a function-blocking mutation (GYG to AYA) in the pore-forming region. As shown in Fig. 3, there was a shift in cell populations such that T_{EM} cells expressing the DN Kv1.1 product decreased in number 1, 2, 3, and 4 wk after transduction, whereas T_{CM} cells increased, relative to the control group. Thus, it appears that the inactivation of Kv1.1 channel function can impair T_{EM} proliferation and/or differentiation that may arise through cell division, either by existing T_{EM} cells or by T_{CM} cells that acquire a T_{EM} phenotype. We did not observe preferential cell death (annexin V and 7-aminoactinomycin D) in either population of cells in the GFP-control and DN Kv1.1-transduced cells.

**Kv1.3 blockade by K⁺ channel blockers consistently suppresses the immune function of chronically activated T_{EM} cells and myelin-specific TCLs**

To further demonstrate the functional dependence of T_{EM} cells on Kv1.3, we generated terminally differentiated T_{EM} cells in vitro using anti-CD3 and anti-CD28 Ab stimulation for 5–8 wk in culture. As shown in Fig. 4A, the repeated stimulation eventually yielded a cell population containing >90% T_{EM} cells, as shown by expression of CD4, CCR7, and CD45RA. These chronically activated T_{EM} cells showed a predominant Th1-polarized response with high IFN-γ and very low IL-5 production (IFN-γ/IL-5 ratio >13; data not shown). The effect of K⁺ channel blockers on the proliferative response of these chronic T_{EM} cells to anti-CD3/CD28 or anti-CD3 alone was subsequently examined by the CFSE assay (Fig. 4, B–D). Following stimulation with either anti-CD3 alone or in combination with anti-CD28 for 5 days, significantly fewer cells proliferated under Kv-blocking conditions as compared with control untreated cells. Cytokine levels were assessed in the supernatants of those cultures by ELISA on day 5. Stimulation of chronic T_{EM} cells with anti-CD3/CD28 induced a significant production of Th1 cytokine IFN-γ, whereas there was no corresponding increase in IL-5, indicative of a Th2 response (data not shown). Addition of K⁺ blockers to the anti-CD3/CD28-stimulated cells resulted in a significant reduction in the production of IFN-γ in comparison with untreated cells (Fig. 4E). These data suggest that all three Kv1.3 channel blockers are capable of suppressing both proliferation and cytokine production of chronic T_{EM} cells.

To further explore the effect of Kv1.3 channel blockers on the proliferation of myelin-specific T cells derived from MS patients, we first established TCLs (with a T_{EM} phenotype) reactive to MBP, and then treated them with channel blockers. Results of these studies show that the proliferation of three different TCLs in response to MBP was quickly and consistently suppressed by ShK (Fig. 4F). In contrast, ShK did not significantly influence the proliferation of other TCLs, such as glatiramer acetate-reactive TCLs (data not shown), which have been shown to be dominated by CCR7⁺ cells (25).

**Discussion**

Results of our studies show that Kv1.3 channel blockade specifically and persistently blocks proliferation and effector responses of T_{EM} cells, which are implicated in pathogenesis of autoimmune diseases, while sparing T_{CM}, which are vital to immunological memory. These data are consistent with previous observations made on short-term T cell subset lines using the patch-clamp technique (4). We evaluated the effects of three potassium channel blockers with varying specificity on T cell proliferation and found that both MgTx and ChTx, which are less selective, potently inhibited cell proliferation and cytokine production in both acutely activated freshly isolated CD4⁺ T cells and chronically activated T_{EM} cells.

ChTx reduced proliferation of CD4⁺ T cells acutely activated with anti-CD3/CD28, predominantly in the T_{CM} cells, which was in contrast to the effect of MgTx on T_{EM}. This differential effect is most likely due to the fact that KCa3.1 is the functionally dominant K⁺ channel in activated T_{CM} cells (1, 4), and thus is the primary target of ChTx in these cells. Thus, because ChTx has a dual specificity for both KCa3.1 and Kv1.3 channels in human T lymphocytes, its antiproliferative effect on the freshly sorted T_{EM} and chronic T_{EM} cells is most likely attributable to its efficient blocking of Kv1.3 channels, because T_{EM} do not express significant levels of KCa3.1.

Interestingly, ShK, which is more selective for Kv1.3, had no significant effect on cell proliferation when a potent costimulatory signal was applied with anti-CD28, even at concentrations as high as 100 nM, but it suppressed cell proliferation in the absence of CD28 costimulation as well as in the chronically activated T_{EM}. Thus, the ability of ShK to effectively suppress anti-CD3-induced proliferation of unsorted CD4 T cells and freshly isolated T_{EM} cells, but its failure to inhibit CD3/CD28-induced proliferation of these cells suggests that CD28-mediated costimulation signals can overcome the blocking effect of ShK on CD3-dependent T cell activation. In contrast to its lack of inhibitory effect on cell proliferation in acutely activated T cells through CD3/CD28, ShK retained its suppressive property on cell proliferation in chronic T_{EM} cells even when activated with anti-CD3/CD28. Because repeated stimulation of T cells and differentiation into T_{EM} lead to
costimulation independence (decreased reliance on CD28 signaling), this finding further supports a selective role for ShK on the CD3 signaling pathway in TEM.

A previous study also found that pharmacological blockade of Kv1.3 had no effect on the alternative T cell activation pathways delivered through CD28 or IL-2 (26). Thus, one potential explanation for our data is that CD28 costimulatory signaling may overcome the known inhibitory effect of Kv1.3 blockade on calcineurin-mediated dephosphorylation of NF-AT and translocation into the nucleus. The principal effect of simultaneous engagement of CD28 has been shown to be an increase in the amplitude of the CD3 transcriptional response by decreased nuclear export of NF-AT (27) or by facilitating phospholipase Cγ1 activation and enhanced calcium flux, leading to increased NF-AT nuclear importation (28). CD28-mediated signals have also been shown to intersect with the TCR/CD3 pathway distally at JNK (29). Thus, partial inhibition of calcium flux by weak pharmacological blockade may be effective in dampening T cell activation, especially in TEM, where alternative costimulatory pathways are less involved. However, it remains unclear why the DN Kv1.1.x-transduced cells showed a markedly significant enrichment of TEM even after stimulation with both anti-CD3 and anti-CD28. This could represent the effects of more complete and sustained inhibition of calcium flux and NF-AT translocation into the nucleus, but alternative signaling pathways may also be involved. The mechanism underlying the effects of DN Kv1.1.x on T cells is actively being explored in our laboratory.

The higher concentrations of potassium channel blockers required in our functional assays than has been noted in electrophysiological assays may relate to the propensity for ShK to bind proteins or be degraded. Although the IC50 for ShK in physiological assays may relate to the propensity for ShK to be degraded in our functional assays than has been noted in electrophysiological studies, this finding further supports a selective role for ShK on the CD3 signaling pathway in TEM.

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Disclosures

Dr. Michael Pennington is the chief operating officer of Bachem Bio-science (manufacturers of ShK); Drs. Pennington and Calabresi are presently unpaid consultants for AirMid.

References


