High-throughput profiling of ion channel activity in primary human lymphocytes

Daniel J. Estes,† Sohiel Memarsadeghi,† Steven K. Lundy,‡ Francesc Marti,† Daniel D. Mikol,† David A. Fox,‡ Michael Mayer*,†,§

†Department of Biomedical Engineering, ‡Department of Internal Medicine and Rheumatic Disease Core Center, †Department of Neurology, §Department of Chemical Engineering
University of Michigan, Ann Arbor, MI, 48109
mimayer@umich.edu

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Supporting Methods

Cell culture and separations. To separate subsets of lymphocytes from whole blood, we first isolated peripheral blood mononuclear cells (PBMCs). We centrifuged whole blood at $700 \times g$ for 20 min to isolate the intermediate “buffy coat” layer containing white blood cells. A subsequent density gradient centrifugation (Histopaque, Sigma-Aldrich, St. Louis, MO) at $700 \times g$ for 20 min allowed separation of PBMCs from this buffy coat layer. Isolated PBMCs were washed twice in wash buffer, defined here as Dulbecco’s modified phosphate buffered saline without Ca$^{2+}$ or Mg$^{2+}$ (D-PBS, Invitrogen, Carlsbad, CA) and supplemented with 2% (v/v) fetal bovine serum (FBS, Mediatech, Herndon, VA). To select for particular subsets of lymphocytes, we separated PBMCs using magnetic beads coated with antibodies against either CD4, CD8, CD19 (for B cells), or the $\gamma\delta$ T cell receptor (Miltenyi Biotec, Auburn, CA). Briefly, we added 50 µL of bead solution to 350 µL of PBMCs suspended in wash buffer and incubated for 15 min at room temperature. We then passed labeled cells through a paramagnetic column (Miltenyi Biotec) and collected positively-labeled cells which were retained in the column. After magnetic separations in columns, we stored cells either at 4° C for flow cytometry analysis or prepared them immediately for high-throughput electrophysiology measurements.

To culture and stimulate cells, we first washed the separated PBMCs in culture media, here defined as RPMI-1640 medium (ATCC, Manassas, VA) supplemented with 10% (v/v) FBS, 150 U mL$^{-1}$ penicillin, 150 µg mL$^{-1}$ streptomycin (pen-strep, Invitrogen), and 55 µM 2-mercaptoethanol (Invitrogen). To stimulate T cells, we added soluble monoclonal mouse anti-human CD3 antibody (clone UCHT1, BD Biosciences, San Jose, CA) at indicated concentrations in culture media. For co-stimulation experiments, we additionally added 150 ng mL$^{-1}$ soluble monoclonal anti-CD28 antibody from mouse (clone CD28.2, BD Biosciences) to PBMCs in suspension. Cells with added antibodies for stimulation were seeded in tissue culture flasks (8 mL into T25 flasks, BD Biosciences) at a concentration of 1×10$^6$ cells mL$^{-1}$ and cultured at 37° C in an atmosphere of 5% CO$_2$, for 72 h unless otherwise noted. After culture, we washed PBMCs twice in wash buffer and then used magnetic bead separations to isolate stimulated CD4$^+$ and CD8$^+$ T cells. These stimulated cells were either stored at 4° C for flow cytometry or prepared immediately for high-throughput electrophysiology.

Wash protocols for the high-throughput electrophysiology instrument. After measuring Kv1.3-specific currents in all 384 wells of the patch plate, we used the automated wash protocols of the IonWorks HT system to flush the internal fluidics and microfluidics array for dispensing drugs to each well of the “patch plate”. The sequence of wash steps consisted of a flush with 5% Hellmanex II (Hellma GmbH & Co. KG, Müllheim, Germany) in H$_2$O followed by a flush with distilled H$_2$O (repeat sequence twice). We also washed the electrode array by soaking the electrode tips in 2.5% Hellmanex II in H$_2$O for 60 s followed by a final soak in D-PBS for 120 s.

Algorithms to quantify Kv1.3-specific currents. We developed custom computer software using the PERL language to quantify specifically the ion current passing through Kv1.3 ion channels. The algorithm first automatically filtered out cells that did not maintain stable seals with the micropore in each well of the patch plate. For a cell to be considered “stable” and to yield a valid whole-cell recording, we imposed two criteria: (i) the cell had to maintain a seal resistance with the micropore of the patch plate of at least 75 MΩ throughout the experiment (including after addition of the compound); and (ii) the magnitude of the seal resistance had to be constant within ± 25% from pre-compound to post-compound measurements.

For cells with valid seals, we quantified Kv1.3-specific currents by subtracting the post-compound (i.e. post blockage with ShK-F6CA) current from the pre-compound current (Supporting Figure 3B). The PERL computer algorithms computed the pre-compound current by determining the maximum current (averaged during a 1-ms “time-window”) after the depolarizing pulse and subtracting an average
of the current before the depolarizing pulse (baseline current). The post-compound current consisted of quantifying, again, the average current over a 1 ms interval. This interval was taken at the exact same time after applying the depolarizing pulse as the time interval from the pre-compound recording. The algorithm then, again, subtracted an average of the current before the depolarizing voltage pulse (baseline current). Finally, the algorithm computed the Kv1.3-specific current from the difference between the pre- and post-compound currents (Supporting Figure S-3). We confirmed the accuracy of these algorithms in each experiment by viewing the largest currents in each experiment graphically, but all computations of currents were performed automatically by the PERL programs to ensure unbiased, reliable, and automated data analysis.

**Flow cytometry assays.** We performed flow cytometry analysis using a four-color flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ) with CellQuest software. Cells were stained with antibodies against the following antigens (all from BD Biosciences) conjugated to either the fluorophore allophycocyanin (APC) or phycoerythrin (PE): CD4-APC, CD8-APC, CD8-PE, CD19-PE, CD25-PE, CD26-PE, CD62L-PE, CD69-PE, and CD69-APC. All staining, incubation (for 30 min), and wash procedures occurred at 4°C using wash buffer. For each type of surface marker, we measured forward scatter, side scatter, and fluorescence intensity from at least 5,000 cells (typically from over 10,000 cells). All cytometer settings (compensation and detector voltages) remained constant for all experiments. We typically quantified mean fluorescence intensity (MFI) for all cells gated (i.e. selected) as lymphocytes based on size (forward scatter) and complexity (side scatter) using analysis software (WEASEL, The Walter and Eliza Hall Institute, Parkville, Australia). For cells stained with CD69, we determined the percentage of cells with “high” levels of CD69 (CD69+) by computing the percentage of cells with fluorescence intensities above the background fluorescence levels of unstained cells.

**CFSE assays to quantify proliferation in CD4+ and CD8+ T cells.** To quantify the proliferation of T cells, we measured the levels of carboxyfluorescein diacetate succinimidyl ester (CFSE) in individual T cells. Labeling cells with CFSE made it possible to determine T cell proliferation by flow cytometry, as cells evenly split their concentration of CFSE when they divided. We performed experiments with cells labeled with CFSE in parallel to experiments with unlabeled cells for high-throughput electrophysiology measurements because electrophysiology experiments required a significantly higher number of cells than CFSE experiments. We stained cells with 0.25 µM CFSE according to instructions from the manufacturer (Invitrogen). Stained populations of cells were seeded into flat-bottom 96-well plates using the same cell density, media, and stimulation conditions as the corresponding cultures for Kv1.3 measurements.

Before analysis, we labeled CFSE-stained cells with either anti-CD4-APC or anti-CD8-APC antibodies to observe specifically the proliferation in those subsets. We used APC-labeled antibodies to avoid “interference” between APC and CFSE (measured using the FITC excitation and detection settings) during the detection by flow cytometry. To quantify proliferation from the resulting flow cytometry data, we performed the following three steps: first, we selected (or gated) for lymphocytes based on the size (forward scatter) and complexity (side scatter) of the cells in the cytometer; second, among gated-lymphocytes, we selected for cells with high levels of APC fluorescence, representing CD4+ or CD8+ T cells; and third, for gated CD4+ or CD8+ T cells, we determined the percentage of cells in each of the clearly-defined CFSE “peaks”. Each peak corresponded to cells that divided 0×, 1×, 2×, 3×, 4×, etc., respectively. Determining the percentages of cells that divided a known number of times allowed calculation of the average number of cell divisions for a specific cell population.

**Statistical Analyses.** We calculated all P-values using a two-sample Student’s t-test (Origin, Northampton, MA). Means and standard deviations are clearly indicated throughout the text, with all standard deviations calculated using sample standard deviation. In indicated cases, we used standard
error of the mean (defined as standard deviation divided by $\sqrt{N}$) to depict potential errors in mean values of Kv1.3 activity determined from individual high-throughput electrophysiology experiments.
Table S-1. Throughput of automated ion channel experiments using lymphocyte subsets isolated from human blood.

<table>
<thead>
<tr>
<th>lymphocyte subset</th>
<th>percentage of wells in the “patch-plate” with successful Kv1.3 measurements, %</th>
<th>average seal resistance of successful cells, MΩ</th>
<th>main reason for unsuccessful wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4$^+$ T cells</td>
<td>42 ± 9</td>
<td>134 ± 21</td>
<td>lost/low seals after compound addition</td>
</tr>
<tr>
<td>CD8$^+$ T cells</td>
<td>32 ± 9</td>
<td>124 ± 23</td>
<td>lost/low seals after compound addition</td>
</tr>
<tr>
<td>B cells</td>
<td>15 ± 6</td>
<td>123 ± 6</td>
<td>lost/low seals after compound addition</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>25 ± 3</td>
<td>116 ± 4</td>
<td>low seal resistance between cell and microwell</td>
</tr>
<tr>
<td>stimulated CD4$^+$ T cells $^*$</td>
<td>46 ± 12</td>
<td>152 ± 23</td>
<td>no seal between cell and microwell</td>
</tr>
<tr>
<td>stimulated CD8$^+$ T cells $^*$</td>
<td>50 ± 13</td>
<td>146 ± 23</td>
<td>no seal between cell and microwell</td>
</tr>
</tbody>
</table>

All subsets measured using half of a 384-well patch-plate.

Values represent average ± standard deviation of mean values from individual subjects involving the indicated subtype ($N = 17$ for CD4$^+$ T cells, $N = 10$ for CD8$^+$ T cells, $N = 3$ for B cells, $N = 3$ for γδ T cells, $N = 10$ for stimulated CD4$^+$ T cells, and $N = 10$ for stimulated CD8$^+$ T cells).

$^*$ T cells stimulated for 3 d with 150 ng mL$^{-1}$ anti-CD3 antibodies.

There may be several reasons for the different throughputs of the various subsets of lymphocytes using the IonWorks HT high-throughput electrophysiology system. Cell size may affect the stability of lymphocytes attached to the micropores of the “patch plate”. Stimulated T cells, with an average of ~1.5 times the diameter of resting T cells from blood, exhibited higher average seal resistances than resting T cells ($p < 0.05$ for both CD4$^+$ and CD8$^+$ T cells). These stimulated T cells were also ~20% less likely than resting T cells to lose their seals after addition of the ShK-F6CA compound to block Kv1.3 channels ($p < 0.01$ for both CD4$^+$ and CD8$^+$ T cells).

A second reason for the different throughputs may be related to surface morphology of the lymphocyte subset. CD4$^+$ and CD8$^+$ T cells isolated from blood, which have very similar sizes and which were always measured in parallel on the same patch plate, exhibited different throughputs in high-throughput electrophysiology experiments. CD4$^+$ T cells showed ~10% higher throughput than CD8$^+$ T cells ($p < 0.01$), as CD4$^+$ T cells were 15% more likely to contact the micropore and 10% more likely to have sufficiently high seals after initially contacting the micropore than CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cells were equally likely to lose seals after compound addition. Properties of CD4$^+$ and CD8$^+$ T cells that may contribute to the initial sealing to the micropore might include differences in phenotypes, expression of certain cell surface molecules, and perhaps differences in mechanical properties.
An interesting finding in the analysis of throughputs of different lymphocyte subsets is that B cells and γδ T cells isolated from blood had significantly lower throughput than CD4+ or CD8+ T cells. γδ T cells were especially likely to form low and insufficient seals with the micropores but otherwise behaved similarly to CD4+ T cells. B cells, which exhibited the lowest throughput of all cell types measured, were the least likely subset to initially contact the micropore and was the most likely subset to lose seals after addition of compound. The reasons for this instability may be related to morphological or mechanical properties of B cells.

Modifications to the patch plates, either by having smaller diameters of micropores or by coating the plates with adhesive molecules, may help to increase seal resistances and stability and increase throughputs for all cell types. The observed differences in behavior of different lymphocytes attached to micropores may, however, allow insights into the mechanical properties of lymphocytes and may provide a platform for rapid screening of drug compounds or molecules that affect the cytoskeleton and mechanical stability of primary cells.
Figure S-1. Confirming full blockage of Kv1.3 ion channels. This graph quantifies the correlation of the recorded whole-cell ion current after addition of ShK-F6CA (post-compound currents) as a function of the ion current before addition of the blocker (pre-compound currents). The recorded currents were obtained from CD8$^+$ T cells from the time-course shown in Figure 5B in the main text (stimulation with both anti-CD3 and anti-CD28 antibodies, number of CD8$^+$ T cells, $N = 342$ from the five time-points). The red line represents a linear best fit of to the data (slope = $-0.0001$, $R^2 = 2 \times 10^{-7}$). The slope of this line was zero, and the absence of correlation of the data indicates that the magnitude of the post-compound currents was not dependent on the magnitude of the pre-compound currents. This absence of correlation demonstrates full-block of Kv1.3 ion channels.
Figure S-2. Specificity of the high-throughput electrophysiology method for Kv1.3 ion channel activity. This figure depicts the blockage of ion current through stimulated CD8+ T cells by different drug compounds. Electrical currents recorded before addition of the drug compound (black line) were compared to currents through the same cell after 3 min incubation with the respective drug compound (red lines). Compounds tested included the following known blockers of Kv1.3 ion channels at concentrations well above their IC$_{50}$ value for Kv1.3 ion channels: (A) 72 nM ShK-F6CA (IC$_{50}$ = 48 pM); (B) 160 nM margatoxin (IC$_{50}$ = 110 pM); (C) 250 nM Psora-4 (IC$_{50}$ = 3 nM), which was initially dissolved in DMSO before dilution in D-PBS. For these compounds, the residual electrical current after addition of the compound (i.e. in the presence of the blocker) was severely attenuated and did not display the time-dependent de-activation that is characteristic of Kv1.3 ion channels. These residual currents after addition of blocker were mostly due to a “leak” current from imperfect electrical seals between the cell and the micropore in the patch plate. A part of these currents may have also been due to the presence of other voltage-gated ion channels than Kv1.3 channels. The following compounds and solutions that are known not to block Kv1.3 ion channels were also tested: (D) D-PBS; (E) 0.33% DMSO in D-PBS; (F) 150 nM TRAM-34, which was initially dissolved in DMSO before dilution in D-PBS. The example of TRAM-34 is particularly important because TRAM-34 blocks the calcium-gated potassium ion channel KCa3.1 (IC$_{50}$ = 20 nM), which is the other significant potassium ion channel in T cells (besides Kv1.3). For each of these conditions, the electrical current after addition of the compounds (or solution) was not attenuated and still displayed time-dependent de-activation of Kv1.3 channels. The observation that TRAM-34 did not block the electrical current through T cells in this assay suggests that most of the K$^+$ current measured in this assay was due to Kv1.3 ion channels. This result, in combination with using the ShK peptide, a blocker highly specific for Kv1.3 channels,
Confirms that the high-throughput assay presented here is specific for Kv1.3 ion channels. Note that all compounds were dispensed using the integrated fluidics of the high-throughput electrophysiology device, and all concentrations of compounds refer to final concentrations in the microwells. All graphs are representative measurements from at least three independent experiments for each compound or solution.

**Figure S-3. Algorithms to quantify Kv1.3-specific currents.** This figure shows a graphical representation of the data processing algorithms used to quantify Kv1.3-specific currents. **(A)** Schematic of the patch-plate with CD4⁺ T cells measured on the left half (192 wells) and CD8⁺ T cells measured on the right half of the plate. Only cells with sufficiently high (>75 MΩ) and stable seals between the cell and micropore were included for analysis (such wells of the micropore are depicted in yellow). Wells in which no cell sealed to the micropore (red squares without an “×”) or wells in which cells did not have sufficiently high or stable seals (red squares with an “×”) were excluded from further analysis. **(B)** Application of a voltage-pulse to quantify the ion currents before (pre-compound current) and after addition of a highly-specific blocker (ShK) of Kv1.3 ion channels (post-compound current). All current amplitudes (represented by the vertical arrows) were obtained by subtracting the current before application of the pulse from the maximum current after addition of the pulse (all current values were obtained by averaging over a 1-ms window). **(C)** Graphical example of a “Kv1.3-specific” current trace, obtained by subtracting the post-compound current from the pre-compound current at each time-point. The values displayed throughout the text, however, represent the pre-compound current minus the post-compound current, as computed in panel (B). All determinations of sufficiently high and stable seals as well as quantifying of amplitudes of Kv1.3-specific currents were computed automatically using custom-written PERL algorithms.
FIGURE S-4

A

Subject 1

Subject 2

Subject 3

mean Kv1.3 activity, nA

0.00 0.15 0.30 0.45 0.60

0 20 40 60 80 100 time after onset of stimulation, h

B

mean FSC

0 20 40 60 80 100 time after onset of stimulation, h

C

% CD69+ cells

0 25 50 75 100%

D

CD25, MFI

0 500 1000 1500 2000

E

CD26, MFI

0 500 1000 1500 2000

F

CD62L, MFI

0 1500 3000 4500 6000

G

avg number cell divisions

0 1 2 3 4

0 20 40 60 80 100 time after onset of stimulation, h
Figure S-4. Individual time-courses of functional Kv1.3 activity after mitogenic stimulation in three different subjects. This figure shows individual time-courses of functional Kv1.3 activity, as well as other T cell parameters, for three subjects following mitogenic stimulation with anti-CD3 antibodies (Fig. 4 in main text). CD4⁰ T cells are depicted as red circles, and CD8⁰ T cells are depicted as black squares. Time-courses for each of the three individual subjects are separated into three columns (all time-courses in the same column are from the same subject), with each row depicting a time-course of a single T cell parameter. (A) High-throughput measurements of mean Kv1.3 currents in T cells from freshly drawn blood (0 h) and 2 h, 24 h, 48 h, 72 h, and 96 h after the onset of mitogenic stimulation of isolated PBMCs with 150 ng mL⁻¹ anti-CD3 antibody. Error bars represent standard error of the mean. (B through G) Flow cytometric analysis of other lymphocyte properties following stimulation. (B) Mean forward scatter (related to cell volume). (C) Percentage of cells expressing high levels of CD69. (D) Mean fluorescence intensity (MFI) of CD25 expression. (E) MFI of CD26 expression. (F) MFI of CD62-L expression. (G) Average number of cell divisions quantified by CFSE dilution.
**FIGURE S-5**

**Figure S-5. Time-course of functional Kv1.3 activity after stimulation with both anti-CD3 and anti-CD28 antibodies.** This figure shows time-courses of functional Kv1.3 activity, as well as other T cell parameters, following stimulation with both anti-CD3 and anti-CD28 antibodies (Fig. 5A,B in the main text). CD4⁺ T cells are depicted as red circles, and CD8⁺ T cells are depicted as black squares. (A) High-throughput measurements of mean Kv1.3 currents in T cells from freshly drawn blood (0 h) and 2 h, 24 h, 48 h, 72 h, and 96 h after the onset of mitogenic stimulation of isolated PBMCs with 150 ng mL⁻¹ anti-CD3 antibody and 150 ng mL⁻¹ anti-CD28 antibody. Error bars represent standard error of the mean. (B) Largest single Kv1.3 current measured, here denoted as “maximum Kv1.3 current”. (C) Average cell diameter, measured immediately prior to high-throughput electrophysiology experiments using a Coulter counter. (D through I) Flow cytometric analysis of other lymphocyte properties following stimulation. (D) Percentage of cells expressing high levels of CD69. (E) Mean fluorescence intensity (MFI) of CD25 expression. (F) MFI of CD26 expression. (G) MFI of CD45-RO expression. (H) MFI of CD62-L expression. (I) Average number of cell divisions quantified by CFSE dilution.
Figure S-6. Distribution of Kv1.3 ion currents in regulatory T cells and dendritic cells. This figure displays histograms of ion current from pilot experiments that measured Kv1.3 activity in CD4+ T cells, regulatory CD4+ T cells (T_{reg}) isolated from human peripheral blood and cultured for 24 h, as well as dendritic cells cultured for 7 d. (A) Cultured CD4+ T cells, \( N = 85 \) total cells measured from one experiment (mean ion current through Kv1.3 ion channels = 0.21 ± 0.11 nA, mean ± standard deviation). (B) Cultured T_{reg} cells, \( N = 32 \) (mean Kv1.3 ion current = 0.13 ± 0.12 nA). Note that the CD4+ T cells in panel (a) and the T_{reg} cells in panel (b) were isolated from the same individual and separated and cultured under the same conditions. T_{reg} cells and CD4+ T cells were initially separated from freshly isolated PBMCs using magnetic bead kits (Miltenyi Biotec, using the CD4+/CD25+/CD127dim- kit for T_{reg}). CD4+ T cells and T_{reg} cells, with magnetic beads still attached, were cultured for 24 h in culture media supplemented with IL-2 (20 U mL\(^{-1}\), Sigma), TGF-beta (2 ng mL\(^{-1}\), Sigma), IL-10 (5 \( \mu \)g ml\(^{-1}\), eBioscience, Inc., San Diego, CA), and anti-CD3 antibodies (clone OKT3, 50 ng mL\(^{-1}\), eBioscience). Note that the culture conditions for the CD4+ T cells shown here and the T_{reg} cells shown here were significantly different from the culture conditions for cells in the main text. Here conditions were used to sustain cells rather than activate them. (C) Dendritic cells, \( N = 67 \) cells from one experiment (mean Kv1.3 ion current = 0.06 ± 0.09 nA). Dendritic cells were prepared by culturing monocytes isolated by a magnetic bead kit (Miltenyi Biotec) in culture media for 7 d in the presence of 20 ng mL\(^{-1}\) GMC-SF (Sigma) and 20 ng mL\(^{-1}\) IL-4 (eBioscience).