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ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2008

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High-Throughput Profiling of Ion Channel Activity in Primary Human Lymphocytes

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We present a high-throughput method to quantify the functional activity of potassium (K^+) ion channels in primary human lymphocytes. This method is rapid, automated, specific (here for the voltage-gated Kv1.3 ion channel), and capable of measuring, in parallel, the electrical currents of over 200 individual lymphocytes isolated from freshly drawn blood. The statistics afforded by high-throughput measurements allowed direct comparison of Kv1.3 activity in different subsets of lymphocytes, including $CD4^+$ and $CD8^+$ T cells, $\gamma\delta$ T cells, and B cells. High-throughput measurements made it possible to quantify the heterogeneous, functional response of Kv1.3 ion channel activity upon stimulation of $CD4^+$ and $CD8^+$ T cells with mitogen. These experiments enabled elucidation of time-courses of functional Kv1.3 activity upon stimulation as well as studies of the effects of the concentration of mitogenic antibodies on Kv1.3 levels. The results presented here suggest that Kv1.3 ion channel activity can be used as a functional activation marker in T cells and that it correlates to cell size and levels of a surface antigen, CD25. Moreover, this work presents an enabling methodology that can be applied widely, allowing high-throughput screening of specific voltage-gated ion channels in a variety of primary cells.

Ion chanels in cells of the immune system serve important roles in generating an effective response to pathogens.[1] The plasma membranes of lymphocytes contain a variety of calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) ion channels that help to regulate at least four cellular properties, including (i) the membrane potential; (ii) signal transduction, especially in the activation of different cell types; (iii) cell volume; and (iv) cell motility.[2]

One of the best studied ion channels in immune cells is the voltage-gated K^+ ion channel, Kv1.3, in human T cells.[3,4] Kv1.3 ion channels regulate the resting potential of T cells and help to regulate Ca^{2+}-signaling after activation of T cells.[5,6] This Ca^{2+}-signaling leads to the expression of cytokines that mediate proliferation and differentiation of effector T cells as well as regulation of the inflammatory response.[7] Interestingly, T cells with high Kv1.3 ion channel activity have been implicated in the pathogenesis of human autoimmune diseases including multiple sclerosis, type I diabetes, and rheumatoid arthritis.[8–10] Therapeutic compounds that specifically block Kv1.3 activity have led to amelioration of the symptoms of the above diseases in animal models.[9,11] The Kv1.3 ion channel is, therefore, a promising therapeutic target for autoimmune diseases,[12–15] and studying the roles and functions of Kv1.3 channels in primary human T cells, as well as other cells of the immune system, is critical for understanding the consequences of therapies that involve blocking Kv1.3 channels.

A significant challenge in studying Kv1.3 ion channels in primary lymphocytes is the lack of an accessible and high-throughput technique to conduct parallel recordings of functional ion channel activity in various subpopulations of cells. Previous

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10.1021/ac800164v CCC: $40.75
Published on Web 04/24/2008

studies have employed manual patch-clamping,[8,16] which involves contacting a single cell with a glass micropipet to gain electrical access to the inside of the cell followed by measuring the flow of ions through the plasma membrane of the entire cell.[17] While this manual whole-cell patch clamping technique is extremely sensitive and versatile, the technique is serial, has low throughput, and requires significant expertise.[18] As a result, quantifying ion channel activity in primary cells of the immune system under various conditions has thus far not been a feasible approach for most immunologists and clinicians.

Here, we present an automated, high-throughput method capable of measuring functional Kv1.3 ion channel activity in over 200 individual lymphocytes within 1 h. In recent years, several new technologies have emerged that automate the patch-clamp method, using different strategies[19–22] and offering varying degrees of throughput (typically 16−384 cells per run). In the present study, we used a high-throughput patch-clamp device capable of measuring up to 384 cells per experiment[23,24] to implement an automated assay that is (i) capable of measuring voltage-gated ion channels in a primary cell type, specifically different subsets of lymphocytes; (ii) specific for endogenous, wild-type levels of Kv1.3 ion channels in primary cells from human subjects; and (iii) able to compare different T cell populations in parallel. We applied this assay to elucidate time-courses of Kv1.3 activity after T cell stimulation as well as the effects of different stimulation conditions on the regulation of the activity of this ion channel. This study presents the first example of functional high-throughput screening of ion channels in a primary cell type and makes it possible to place the upregulation of Kv1.3 activity into the context of the signaling cascade during activation of human T cells.

EXPERIMENTAL SECTION

Human Subjects. We obtained peripheral venous blood from 12 healthy subjects without history of autoimmune disease. We obtained multiple blood samples from six subjects. The Institutional Review Board at the University of Michigan approved blood draws and protocols.

High-Throughput Electrophysiology Assay. We used an IonWorks HT high-throughput electrophysiology instrument (MDS Analytical Technologies, Sunnyvale, CA) made available via collaboration with the original developers of the technology (Essen Instruments, Ann Arbor, MI) to measure Kv1.3 currents in lymphocytes. Separated lymphocytes were suspended in Dulbecco's phosphate buffered saline (D-PBS, with Ca^{2+} and Mg^{2+}) at a concentration of 5×10^5 cells mL^{-1} and stored at room temperature for 30 min prior to electrophysiology experiments. A typical experiment required 2.2 mL of cell suspension. Integrated fluidics within the IonWorks instrument then automatically dispensed cells into the single-use 384-well "patch plate". The bottom of these plates consisted of microfabricated polyimide that contained one micropore in each well. For experiments with lymphocytes, we typically used only patch plates with an open hole resistance (i.e., before adding cells) of at least 3.0 MΩ (corresponding to hole diameters below 1.8 μm, measured in D-PBS with Ca^{2+} and Mg^{2+}). In each experiment, $CD4^+$ T cells occupied half of the patch plate (192 microwells) and the other half was occupied by either $CD8^+$ T cells, B cells, or $\gamma\delta$ T cells, allowing for direct comparisons between subsets of lymphocytes. The IonWorks HT instrument applied and maintained suction from below each well to seal a single lymphocyte over the hole in each well of the patch plate. The intracellular recording solution consisted of 100 mM K^+ D-gluconic acid, 50 mM KCl, 3 mM $MgCl_2$, and 5 mM EGTA pH 7.3 (all from Sigma-Aldrich). To gain electrical access to the interior of the sealed cells, automated fluidics introduced a pore-forming peptide (amphotericin B, Sigma-Aldrich, 100 μg mL^{-1} with 0.2% DMSO) in the intracellular recording solution. Recordings started 5 min after introduction of the peptide.

To record Kv1.3 ion channel currents, T cells were first voltage-clamped at a holding potential of −80 mV for 30 s before applying a depolarizing step pulse from −80 mV to +40 mV for 300 ms. This voltage-step opened voltage-gated channels, and the resulting current was recorded in parallel using an array of 48 Ag/AgCl electrodes. After performing this process in all of the 384 wells, the integrated fluidics array added a specific blocker of Kv1.3 channels, 6-FAM-AEEAc-*Stichodactyla helianthus* neurotoxin peptide (ShK-F6CA, Bachem Biosciences, King of Prussia, PA), to a final concentration of 72 nM to each well of the patch plate.[25] After an incubation time of 3 min to specifically block all Kv1.3 ion channels, electrical currents from the attached cells were then measured again using the same voltage protocols as described above. We defined Kv1.3 current as the difference between the pre- and postcompound currents (see Supporting Information Figure S-3 for detailed information on data processing algorithms as well as Supporting Methods for details on wash protocols, cell separations and culture, flow cytometry assays, and statistical analyses).

RESULTS

Automated High-Throughput Profiling of Kv1.3 Ion Channels in Human Lymphocytes. To establish a method that enables rapid profiling of Kv1.3 ion channel activity in different subtypes of lymphocytes from human blood, we adopted a high-throughput patch-clamp technology that had previously been developed for drug-screening in the pharmaceutical industry (Figure 1).[23,24] This technology is based on 384 microwells with a single micropore at the bottom of each well (Figure 1A). Cells in suspension are automatically positioned onto these micropores by suction, enabling up to 384 whole-cell patch-clamp recordings

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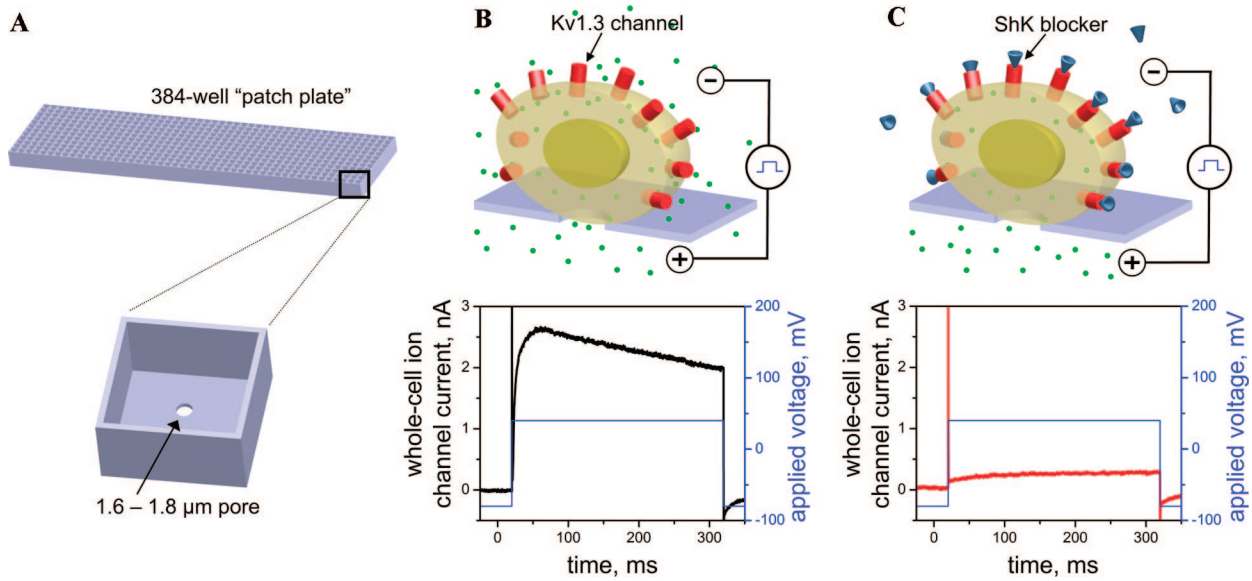

Figure 1. High-throughput method for measuring Kv1.3 ion channel activity in lymphocytes. (A) Isolated lymphocytes in suspension (5×10^5 mL^{-1}) were dispensed into a 384-well patch-plate (1200–2500 cells per well). The bottom of each well contained a micropore with a diameter of 1.6−1.8 μm. Suction resulted in positioning of a single lymphocyte over the hole in each well to form a stable electrical seal (>75 MΩ) with the "patch-plate". (B) After electrical access to the cytoplasm was obtained, a depolarizing voltage step function from −80 to +40 mV was applied across the cell membrane. This depolarization opened voltage-gated ion channels, and the resulting whole-cell current through the plasma membrane was recorded. (C) While the same cell was still attached, a specific blocker of Kv1.3 channels was added to each microwell. A second depolarizing step was applied to the cell, and the resulting whole-cell current was recorded. The Kv1.3-specific current was defined as the difference between the (B) pre-blocker and (C) post-blocker electrical currents.

within 1 h. An integrated microfluidics array dispenses cells, drugs, or other molecules in solution to each of the 384 wells, while keeping the cells attached to the micropore. Previous applications of this high-throughput electrophysiology technology employed cell lines (diameter typically > 15 μm) that overexpressed one particular, recombinant ion channel protein.[26] To the best of our knowledge, there have been no published manuscripts of high-throughput measurements of ion channels in primary cells, possibly because of difficulties in isolating primary cell types in large numbers and possibly because the number of a particular endogenous ion channel in wild-type primary cells may have been considered too low to detect. We chose lymphocytes as examples of primary cell types because (i) the molecular mechanisms of activation of these cells are fundamentally important for an effective immune response; (ii) these cells can be obtained in high yield from peripheral blood; (iii) they can be easily activated ex vivo; and (iv) emerging evidence identifies ion channels in lymphocytes as potential drug targets for treatment of autoimmune disease. These cells, however, offer several challenges to high-throughput screens because of their small sizes (~8 μm diameter for T cells from blood[27]) and diversity with respect to endogenous ion channels in their plasma membranes.[2]

To adapt high-throughput patch-clamp technology for a specific ion channel (here the voltage-gated potassium channel Kv1.3) in subsets of lymphocytes without overexpressing the channel protein, we implemented the following four strategies were implemented. First, we isolated specific lymphocyte subsets ($CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$ T cells, and B cells) using commercially available magnetic beads coated with an antibody against particular surface markers (e.g., CD4) which are expressed on the blood cell subset of interest. Second, we specifically selected 384-well "patch-plates" with micropore sizes below 1.8 μm (Figure 1A). Third, we developed a differential measurement of electrical current through individual cells before and after application of a highly specific blocker of Kv1.3 ion channels (the ShK-F6CA peptide).[25,28] This strategy ensured specificity for the Kv1.3 ion channel while subtracting possible "leak" currents (Figure 1B,C). We confirmed specificity, as well as efficacy of block, by testing additional compounds that either block Kv1.3 ion channels (margatoxin and Psora-4)[12] or have minimal effect (TRAM-34)[12] on these channels (Supporting Information Figures S-1 and S-2). And fourth, we used data processing algorithms (i) to exclude cells that did not remain attached to the micropore throughout the experiment and (ii) to compute the magnitude Kv1.3-specific currents after the differential measurements (Supporting Information Figure S-3).

This high-throughput assay enabled us to profile Kv1.3 activity rapidly in four different subsets of lymphocytes isolated directly from fresh human blood (Figure 2). In each experiment, we measured two subsets on the same patch-plate (all cells were seeded at the same concentrations), with $CD4^+$ T cells on one-half (192 wells) and either $CD8^+$ T cells, $\gamma\delta$ T cells, or B cells on the other half (see Supporting Information Table S-1 for comparisons and discussions of throughput, which can depend on lymphocyte subtype). Examining Kv1.3 currents, we found that although the mean Kv1.3 currents of each of the four lymphocyte

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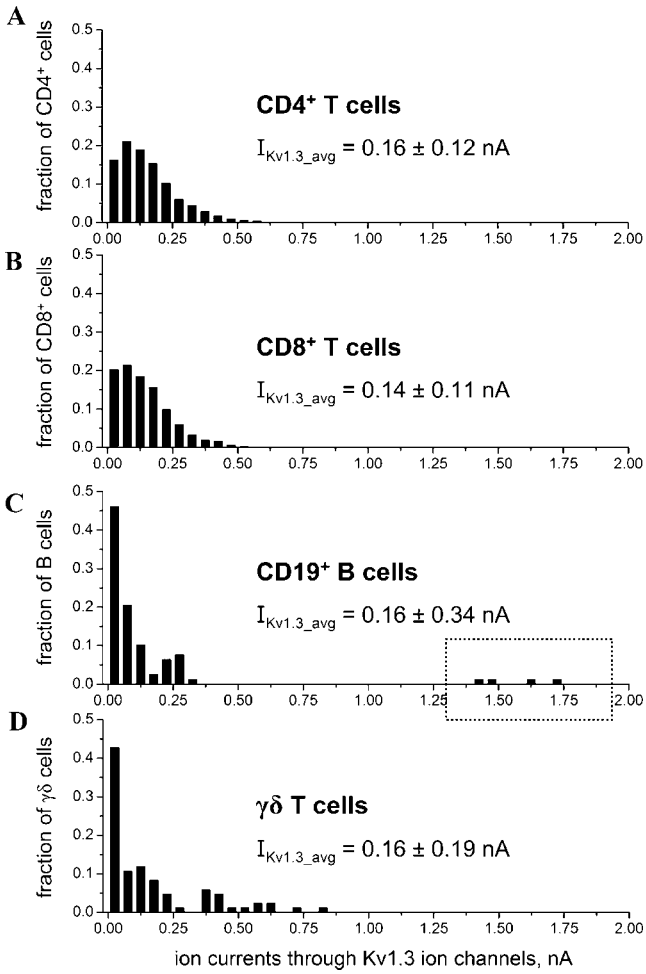

Figure 2. Histograms of current through Kv1.3 ion channels in lymphocyte subsets isolated directly from human blood. Histograms represent the combined totals of Kv1.3 measurements on individual cells isolated from three or more independent blood draws for each subtype. (A) $CD4^+$ T cells, $N = 1220$ total cells measured from 17 independent experiments. (B) $CD8^+$ T cells, $N = 556$ cells from 10 independent experiments. (C) $CD19^+$ B cells, $N = 78$ cells from 3 independent experiments. The dashed box indicates the ~5% of measurements with large Kv1.3 currents above 1.25 nA. These B cells with large Kv1.3 currents were present in two out of the three subjects examined. (D) $\gamma\delta$ T cells, $N = 84$ cells from 3 independent experiments. All histograms were constructed with a bin-size of 0.05 nA, and all *y*-axes display the fraction of cells of that subtype (e.g., $CD4^+$ T cells) with Kv1.3 currents in the specified bin. Average Kv1.3 currents ($I_{Kv1.3\_avg}$) for each subtype are listed within each histogram as mean ± standard deviation and were computed using the total number of cells for that cell type.

subtypes were similar, histograms representing the distribution of Kv1.3 currents through 78−1220 individual cells showed significant differences between subtypes (Figure 2). $CD4^+$ and $CD8^+$ T cells displayed similar distributions of Kv1.3 currents; for both of these T cell types, more than 98% of whole-cell currents had a magnitude between 0 and 0.5 nA (Figure 2A,B). In contrast, the majority of B cells displayed Kv1.3 currents below 0.2 nA; however, ~5% of B cells exhibited very high Kv1.3 activity (currents >1.25 nA) (Figure 2C). These rare, large currents in B cells were the largest Kv1.3 currents that we observed from all subsets of primary cells isolated from freshly drawn blood. These cells presumably represented activated class-switched IgD^- $CD27^+$ B cells that express large numbers of Kv1.3 channels.[29]

$\gamma\delta$ T cells, which previously had not been characterized with respect to Kv1.3 ion channel activity, displayed distributions of Kv1.3 currents more similar to B cells than $CD4^+$ or $CD8^+$ T cells (Figure 2D). The majority of $\gamma\delta$ T cells displayed little or no Kv1.3 current, while ~10% of $\gamma\delta$ T cells displayed currents larger than 0.5 nA. These results demonstrate that the high-throughput assay presented here makes it possible to rapidly characterize cells with heterogeneous phenotypes (e.g., B cells) as well as to elucidate differences in Kv1.3 activity among various subsets of lymphocytes.

Quantification of Ion Current through Kv1.3 Ion Channels in Activated T Cells. To validate the high-throughput assay presented here, we measured the increase of Kv1.3 activity in $CD4^+$ and $CD8^+$ T cells after immunologic stimulation. Wulff et al. indicated that T cells increased the number of functional Kv1.3 ion channels after mitogen stimulation with anti-CD3 antibodies, although the degree of upregulation depended on the phenotype of T cell.[8] Naïve and central memory T cells modestly increased Kv1.3 activity 1.5-fold, while effector memory T cells (T_{em}) upregulated Kv1.3 channels 5-fold.[8] The reasons for this increase in functional activity of Kv1.3 ion channels are not well understood but may be related to regulation of cytokine-specific Ca^{2+} signals in activated T cells.[8]

Using the high-throughput method to compare $CD4^+$ and $CD8^+$ T cells in parallel, we found that the mean whole cell currents through Kv1.3 channels in T cells isolated from 10 independent blood draws increased significantly after 72 h of mitogen stimulation with anti-CD3 antibodies (Figure 3). The mean Kv1.3 activity from individual subjects increased by a factor of 3.0 ± 1.5 in $CD8^+$ T cells (mean ± standard deviation) after mitogenic stimulation compared to currents from freshly drawn blood ($P = 0.000\,01$). Correspondingly, mean Kv1.3 activity increased by a factor of 2.5 ± 0.8 in $CD4^+$ T cells ($P = 0.000\,01$). The mean Kv1.3 activities in $CD4^+$ and $CD8^+$ T cells increased to similar extents upon stimulation ($P = 0.20$). Figure 3 shows histograms of Kv1.3 currents from individual cells combined from all 10 experiments from T cells from freshly drawn blood (797 $CD4^+$ and 541 $CD8^+$ T cells) and from T cells after stimulation (823 $CD4^+$ and 888 $CD8^+$ T cells). These histograms show an increased dispersion in Kv1.3 currents after stimulation and highlight the advantages of using high-throughput methods to profile the heterogeneous response of primary T cells to stimulation.

In addition to heterogeneity of responses of individual cells with respect to Kv1.3 activity, high-throughput functional ion channel recording revealed that blood samples from different individuals (i.e., different human subjects) exhibited surprisingly varied Kv1.3 responses to mitogen stimulation (Figure 3A). The largest increase in mean Kv1.3 activity that we found in a sample from one human subject was a 5.7-fold increase in $CD8^+$ T cells; the same subject exhibited a 3-fold increase in $CD4^+$ T cells. In contrast, another subject actually exhibited a slight decrease in Kv1.3 activity in $CD8^+$ T cells after stimulation. T cells from this subject did not exhibit other characteristics of stimulation such as an increase in cell size or upregulation of CD25 or CD26 cell surface markers, indicating a deficient response to the activating mitogen.

Control experiments with blood samples from all subjects in which we cultured T cells without stimulation (i.e., without addition of anti-CD3 antibody) showed no significant increase in

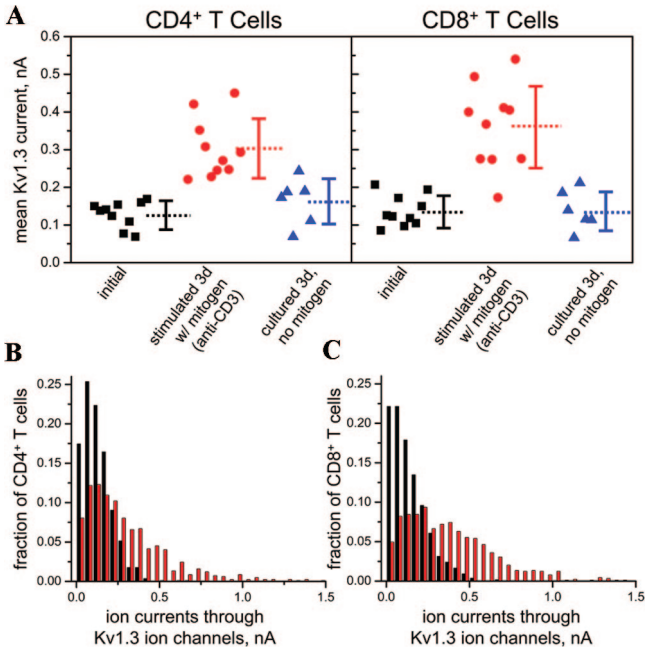

Figure 3. Functional increase of Kv1.3 ion channel activity in stimulated T cells from 10 independent blood draws. (A) Kv1.3 activity was quantified from freshly drawn blood (labeled "initial", ■), after 72 h culture in the presence of 150 ng mL^{-1} anti-CD3 antibody (●), and after 72 h culture without added antibody (no stimulation, ▲). Each point represents mean Kv1.3 current in $CD4^+$ (left) or $CD8^+$ (right) T cells from an individual subject. Dashed lines represent averages of the 10 experiments ($N = 6$ for T cells cultured without stimulation), and the error bars show standard deviation. (B) Histograms of Kv1.3 currents from 797 $CD4^+$ T cells before (black bars) and from 823 $CD4^+$ T cells after 72 h culture with anti-CD3 antibody (red bars). Plots depict the fraction of cells in each histogram bin (bin-size = 0.05 nA). (C) Histograms of Kv1.3 currents from 541 $CD8^+$ T cells before (black bars) and from 888 $CD8^+$ T cells after stimulation (red bars).

Kv1.3 activity in either $CD4^+$ or $CD8^+$ T cells ($N = 6$, $P = 0.19$ for $CD4^+$ T cells, $P = 0.97$ for $CD8^+$ T cells) (Figure 3A). In summary, these results demonstrate three interesting points: (i) the increase in Kv1.3 activity is a direct result of T cell stimulation; (ii) $CD4^+$ and $CD8^+$ T cells responded similarly to stimulation despite differences in clonality and phenotypes;[30,31] and (iii) the magnitude of Kv1.3 increase measured using high-throughput electrophysiology varies by individual but generally agrees with previous measurements using traditional patch-clamping.

Time-Course of Kv1.3 Ion Channel Regulation after T Cell Stimulation. To demonstrate the benefits of the assay presented here, we elucidated time-courses of the functional Kv1.3 ion channel activity in T cells after stimulation. Previous studies have employed manual patch-clamping to study the time-dependent changes of Kv1.3 activity after mitogenic stimulation in a variety of cell types.[25,32–34] Here, high-throughput screening provided additional insight into the time-courses of Kv1.3 activity in stimulated human lymphocytes by providing a large number of measurements within 1 h for statistical analyses and by comparing directly $CD4^+$ and $CD8^+$ subsets during the same experiment (i.e., on the same patch-plate). We conducted time-course studies of three subjects who responded strongly to stimulation with anti-CD3 antibodies (results from these three subjects are shown in Figure 3 with the highest Kv1.3 activity in $CD8^+$ T cells). We measured Kv1.3 activity in $CD4^+$ and $CD8^+$ T cells before, as well as 2 , 24, 48, 72, and 96 h after stimulation with anti-CD3 antibodies (Figure 4A).

Three aspects of this time-course are remarkable. First, 2 h after stimulation, the mean Kv1.3 currents decreased by ~50% compared to initial values from blood. This reduction in activity possibly resulted from phosphorylation of Kv1.3 channels immediately after activation which has been reported to decrease activity.[35] Second, maximum Kv1.3 activity occurred 48 h after stimulation for two subjects and after 72 h for the third subject (mean $CD8^+$ activity increased (4.3 ± 1.2)-fold, $CD4^+$ activity increased (3.6 ± 0.5)-fold). Third, we observed no significant differences between $CD4^+$ and $CD8^+$ T cells in average Kv1.3 currents at any time-point (comparing mean values obtained from the three subjects at each time-point, minimum P value = 0.19).

To compare the changes in Kv1.3 ion channel activity with changes in other parameters of T cells, we measured levels of cell surface markers, proliferation, and cell size at each time-point (Figure 4B–F and Supporting Information Figure S-4). The cell surface marker that correlated most strongly with Kv1.3 was CD25, i.e., the α-chain of the IL-2 receptor. Peak expression of CD25 occurred at the same time-point as peak Kv1.3 activity, although CD25 was upregulated to a greater extent than Kv1.3 ion channels (~30-fold increase in mean fluorescence intensity). Forward scatter, which is related to cell volume, also correlated strongly with the time-course of Kv1.3 activity. The time-courses of other cell surface markers that are commonly used as activation markers, such as CD69, CD26, and CD62L, all displayed distinctly different kinetics of regulation compared to Kv1.3 (Figure 4D,F and Supporting Information Figure S-4). Proliferation data, measured in a parallel assay using carboxyfluorescein diacetate succinimidyl ester (CFSE) to quantify the number of divisions of T cells, indicated that T cell proliferation of both $CD4^+$ and $CD8^+$ T cells initiated between 48–72 h after the onset of stimulation. Hence, proliferation started shortly after the cells reached maximum Kv1.3 activity (Figure 4C). The high-throughput assay presented here, therefore, made it possible to place Kv1.3 activity in the chronology of events during T cell activation.

Effects of Costimulation on Levels of Functional Kv1.3 Ion Channel Activity. As mentioned previously, the data shown in Figure 4 summarized the response in Kv1.3 activity of three subjects who responded strongly to stimulation with anti-CD3 antibodies alone. We also observed subjects who responded weakly to this stimulation (Figure 3A). A reason for these differences may be that T cells require two signals for effective

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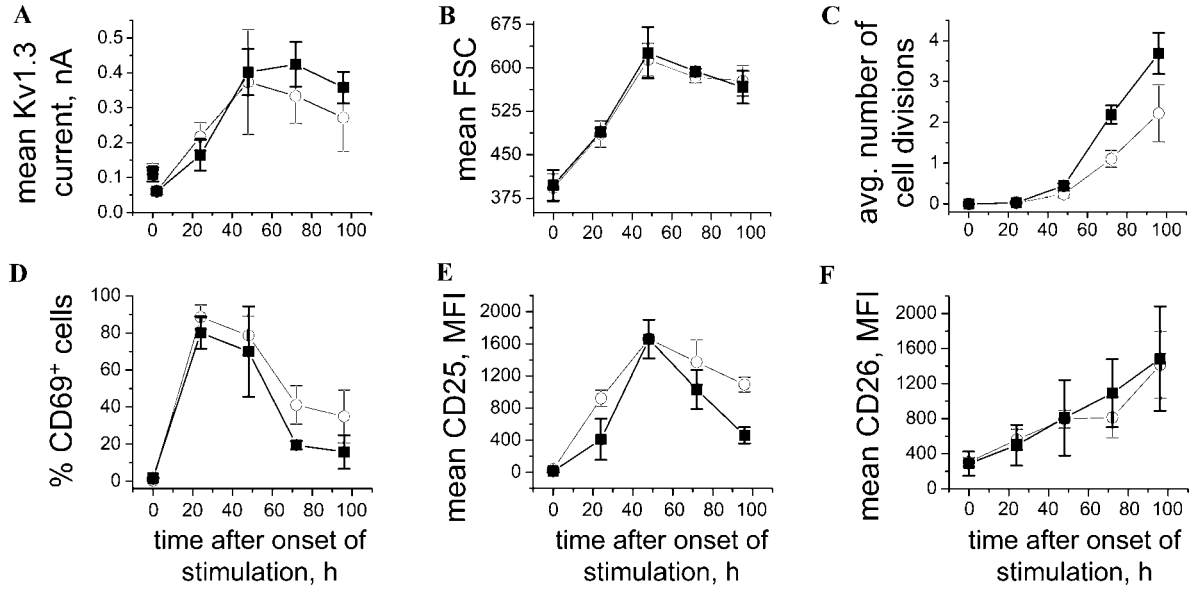

Figure 4. Time-courses of functional Kv1.3 activity, as well as other T cell parameters, following stimulation with anti-CD3 antibody averaged from three subjects. $CD4^+$ T cells are depicted as open circles, and $CD8^+$ T cells are depicted as black squares. (A) High-throughput measurements of mean Kv1.3 currents in T cells from freshly drawn blood (0 h) and 2, 24, 48, 72, and 96 h after the onset of mitogenic stimulation of PBMCs with 150 ng mL^{-1} anti-CD3 antibody. At least 40 $CD4^+$ and 35 $CD8^+$ T cells were averaged at each time point for each subject. (B−F) Flow cytometric analysis of other lymphocyte properties following stimulation. (B) Mean forward scatter (related to cell volume); (C) average number of cell divisions quantified by CFSE dilution; (D) percentage of cells with high expression of CD69; (E) mean fluorescence intensity (MFI) of CD25 expression; (F) MFI of CD26 expression. All points represent averages of three independent experiments (each experiment was performed on a blood sample from a different subject), and the error bars show standard deviation.

stimulation, one through the T cell receptor (TCR) and the second through a costimulatory molecule such as CD28.[36] In experiments without costimulatory anti-CD28 antibodies (Figures 3, 4), T cells presumably received costimulatory signals from other cells in culture. We hypothesize that the heterogeneity of Kv1.3 activity in T cells upon stimulation may in part be due to varying efficiencies of costimulation by other cells in the peripheral blood mononuclear cell (PBMC) population. An advantage of the presented method is that the effects of different stimulation conditions on Kv1.3 activity can be elucidated rapidly and reliably.

To explore the effects of costimulation, we acquired time-courses of Kv1.3 activity after the onset of stimulation with and without added anti-CD28 antibodies from a subject who responded weakly to stimulation with anti-CD3 antibodies alone (Figure 5A,B). Without added anti-CD28 antibodies, Kv1.3 currents increased gradually after stimulation; $CD4^+$ currents peaked 72 h after stimulation, and $CD8^+$ currents peaked 96 h after stimulation. When we added anti-CD28 antibodies during stimulation of T cells from the same subject, we observed markedly accelerated kinetics of Kv1.3 activity. The Kv1.3 current peaked 48 h after stimulation for both $CD4^+$ and $CD8^+$ T cells (Figure 5A,B), and the time-dependent profiles in Kv1.3 activity were now similar to those changes of subjects who responded strongly to mitogen stimulation alone (Figure 4A). Changes in other T cell properties, such as kinetics of expression of cell surface markers, cell size, and proliferation with added anti-CD28 also showed similar correlations to Kv1.3 activity as those of subjects that responded strongly to stimulation by anti-CD3 antibodies alone (Figure 4 and Supporting Information Figure S-5).

To explore the effects of costimulation on upregulation of Kv1.3 further, we investigated the effects of the concentration of added anti-CD3 antibodies on functional Kv1.3 increase after 48 h of stimulation in the presence or absence of a fixed concentration of anti-CD28 antibodies (Figure 5C,D). The subject examined had previously responded strongly to stimulation with anti-CD3 antibodies alone (Figure 3). We observed that maximum Kv1.3 activity occurred at intermediate concentrations of added anti-CD3 antibodies (50 and 150 ng mL^{-1}) and that adding anti-CD28 antibodies increased Kv1.3 currents by ~30% at these concentrations of anti-CD3 antibodies (Figure 5C,D). Stimulation with 150 ng mL^{-1} anti-CD28 antibodies alone did not result in increases in Kv1.3 activity (Figure 5C,D). Interestingly, $CD4^+$ T cells exhibited smaller increases in Kv1.3 activity than $CD8^+$ T cells after stimulation with low (5 ng mL^{-1}) and high (1000 ng mL^{-1}) concentrations of anti-CD3 antibodies. These results suggest that the increase in Kv1.3 channel activity after stimulation depends strongly on the type and intensity of this stimulation. These results also identify stimulation conditions (antibody concentrations, time-course) for obtaining T cells with maximum Kv1.3 activity. Such T cells might be useful to examine potential drug candidates that block Kv1.3 channels.[37]

DISCUSSION

The expression and function of ion channels in lymphocytes is not only dependent on the phenotype of cells but also on their activation status. This characteristic makes ion channels especially attractive as selective pharmaceutical targets. In this context, growing evidence supports that the activity of ion channels is compromised in different immune-related pathologies;[10,38] there are, however, inherent difficulties to measure their functional

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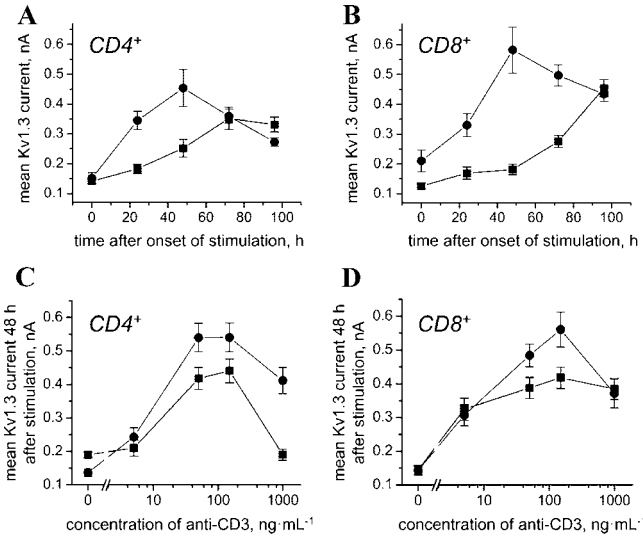

Figure 5. Functional increase of Kv1.3 ion channel activity in mitogen stimulated T cells with or without a costimulatory molecule (anti-CD28 antibody). (A) For a subject with a previously observed weak response to stimulation with anti-CD3 antibodies alone, time-courses of Kv1.3 activity in $CD4^+$ T cells were obtained after stimulation of PBMCs with 150 ng mL^{-1} anti-CD3 antibodies alone (■) or with anti-CD3 plus 150 ng mL^{-1} anti-CD28 antibodies (●). (B) Time-courses of Kv1.3 activity in $CD8^+$ T cells in the same experiment as described in part A. (C) For a subject with previously measured strong response to anti-CD3 stimulation alone, Kv1.3 activity in $CD4^+$ T cells was measured after 48 h of stimulation of PBMCs in the presence of increasing concentrations of anti-CD3 antibodies with (●) and without (■) 150 ng mL^{-1} anti-CD28 antibody. (D) Kv1.3 activity in $CD8^+$ T cells in the same experiment described in part C. All points represent averages of Kv1.3 currents from at least 60 T cells; error bars show standard error of the mean.

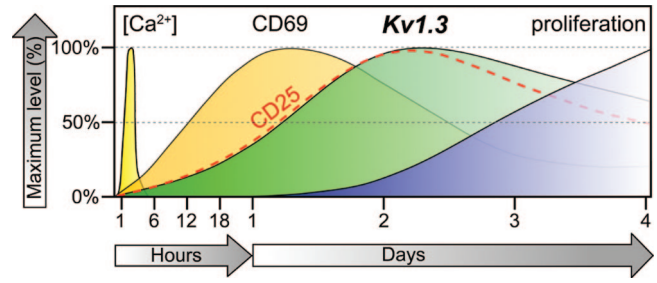

Figure 6. Time-course of functional Kv1.3 activity in the context of increases of other activation markers in T cells. The time-course for Ca^{2+}-signaling (here represented as a spike in the intracellular concentration of Ca^{2+}) was inferred from Lewis.[7] Data for CD69 expression, Kv1.3 activity, CD25 expression, and T cell proliferation were averaged from the three time-courses shown in Figure 4 and from the time-course with added anti-CD28 antibodies in Figure 5. For each individual time-course, values were first averaged between $CD4^+$ and $CD8^+$ T cells and normalized between 0% and 100% to account for differences in peak levels between different subjects. Figure inspired by Abbas and Lichtman.[27]

activity in freshly isolated cells. Here, we introduce a novel high-throughput method that makes the study of specific ion channels in cells of the immune system accessible to biochemists, clinicians, and immunologists who often lack the equipment and expertise for measuring ion channel activity.

This high-throughput assay offers at least three advantages over traditional patch-clamp techniques. First, it affords a significantly increased number of measurements of electrical whole-cell ion currents through individual cells compared to existing techniques. We measured as many as 974 lymphocytes within 10 h, a throughput which is 10−50-fold higher than what is possible with manual patch-clamp techniques. Large numbers of measurements are particularly important in profiling ion channel activity in cell populations with heterogeneous phenotypes such as activated $CD4^+$ and $CD8^+$ T cells. Moreover, high-throughput measurements allow the detection of small subsets of cells within a population that possess high ion channel activity (e.g., B cells or $\gamma\delta$ T cells directly isolated from blood).

Second, the high-throughput assay presented here is rapid (<1 h) and proceeds in a completely automated fashion, including dispensing of cells, establishing seals, performing whole-cell voltage-clamp recordings, and processing data. Third, this assay allows direct comparisons of subsets of lymphocytes under the same experimental conditions and at the same time-points by examining multiple subsets of cells on the same patch-plate. By examining Kv1.3 ion channel activity in $CD4^+$ T cells, $CD8^+$ T cells, and B cells isolated from human blood by magnetic bead separations, we found that the results obtained from the high-throughput electrophysiology method agreed well with previously reported data (Figure 2), confirming the reliability and robustness of this method. These experiments also revealed that $\gamma\delta$ T cells display a different distribution of Kv1.3 activity than do $CD4^+$ or $CD8^+$ T cells (Figure 2). These $\gamma\delta$ T cells had previously not been characterized with respect to the expression of active Kv1.3 channels. $\gamma\delta$ T cells display specific signaling events that differ from other subsets of lymphocytes.[39,40] The results presented here suggest that altered K^+ signaling may be one of these different signaling events.

With respect to immunological implications, we demonstrate the feasibility of measuring Kv1.3 ion channel activity as a functional activation marker of T cells. We demonstrated that levels of ion currents through Kv1.3 ion channels in human T cells peaked between 48 and 72 h after the onset of mitogenic stimulation and more than tripled compared to baseline levels in both $CD4^+$ and $CD8^+$ T cells (Figures 4 and 5, and Supporting Information Figures S-4 and S-5). This increase in Kv1.3 activity depended on both the primary stimulatory signal through the TCR as well as costimulatory signals. The time-course of Kv1.3 activity upon stimulation correlated strongly with expression of CD25 and the sizes of T cells (Figure 6). Maximum Kv1.3 levels also occurred shortly before significant proliferation of T cells (Figure 6). The immunological basis for the functional increase in Kv1.3 activity after stimulation is not well-understood; however, the results presented here, especially the coupling of maximum Kv1.3 activity to the onset of proliferation, suggest that the increase in functional levels of Kv1.3 may be cell-cycle related, perhaps to ensure proper regulation of the resting potential in daughter cells after division.[33]

While the present study focused on lymphocytes, the developed high-throughput methodology could in principle be used to screen functional activity of voltage-gated ion channels in a range of cell types (e.g., other primary cell types or malignant cells, such

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as lymphoma cells, etc.). For instance, we performed pilot experiments that profiled ion channel activity in regulatory T cells (T_{reg}) and dendritic cells (Supporting Information Figure S-6). The requirements for such assays are (i) preparing a primary cell type in suspension that seals well to the planar substrate of the high-throughput patch-clamp device and (ii) identifying a specific blocker of the voltage-gated channel of interest. Several factors affect the sealing of cells to the substrate,[41] most notably cell size in relation to the size of micropore in the patch plate (small cells require small pores). In addition, surface properties of the planar substrate may contribute; modifying the pore size or coating the substrate with adhesive molecules may further increase the number of primary cells that can be analyzed successfully in each experimental run. With regards to blockers of ion channels, a selection of compounds are available that specifically block various K^+, Na^+, Cl^-, and Ca^{2+} channels.[12]

High-throughput profiling of ion channels has three limitations compared to traditional techniques. First, patch-clamping allows for measurements of electrical currents through single ion channels (so-called single-channel recordings), mainly because of the tight electrical "seals" between the tip of a glass pipet and the attached cell (i.e., GΩ seal).[17] In contrast, the instrument used in this work typically operates with seal resistances between 75 and 300 MΩ. Potential leak currents are, however, subtracted by the leak current compensation of the instrument and by taking differential measurements before and after addition of an ion channel blocker.[23] Second, the high-throughput instrument used in this work is not configured to perform measurements of ligand-gated ion channels (some existing automated patch-clamp technologies do allow for measurements of this channel type, albeit at lower throughput[42]). Finally, automated patch-clamp instruments are currently not widely accessible and are typically expensive. Rapid advances in microfabrication,[43] microfluidics, and automated patch-clamp recordings are likely to reduce cost and increase the accessibility to these instruments in the near future.[42,44,45]

CONCLUSIONS

The functional, high-throughput assay presented here provides a tool to investigate the role of ion channels in primary cells, under conditions with different degrees of activation, and in physiological or pathological conditions. Importantly, this method makes it possible to elucidate profiles of the distribution of ion channel activity. We expect this method to serve as a useful and accessible tool for biochemists, cell biologists, immunologists, and clinicians to rapidly and conveniently study ion channel activity in a range of primary cell types. The ensuing insight based on functional activities of ion channel proteins that are involved in cell signaling and immune cell activation may lead to a better understanding of the role of ion channels in physiology, immune responses, and human disease.

ACKNOWLEDGMENT

D.J.E. and S.M. contributed equally to this paper. We thank Kathy Bennett for performing all blood draws; Kirk Schroeder, Eric W. Endsley, John N. Rauch, and Libby Oupicka for assistance with high-throughput electrophysiology experiments; and Jeffrey D. Uram and Ricardo F. Capone for helpful discussions and assistance in preparing samples. This work was supported by the Michigan 21st Century Jobs Fund (M.M., S.K.L., F.M.), NSF (Grant 02-111 CAREER Award, M.M), NIH (Grant 5R03AR052904-02 NIAMS, F.M), and University of Michigan startup funds. D.J.E. acknowledges a fellowship from the Cellular Biotechnology Training Program (CBTP) from NIH and a Rackham Pre-Doctoral Fellowship from the University of Michigan.

SUPPORTING INFORMATION AVAILABLE

Methods, seven figures providing additional details and validations of the assay, and a table detailing the throughput of the presented assay for different lymphocyte subsets. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review January 22, 2008. Accepted March 10, 2008.

AC800164V

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