K⁺ Channel Expression during B Cell Differentiation: Implications for Immunomodulation and Autoimmunity

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K⁺ Channel Expression during B Cell Differentiation: Implications for Immunomodulation and Autoimmunity

Heike Wulff, Hans-Günther Knaus, Michael Pennington, and K. George Chandy

Using whole-cell patch-clamp, fluorescence microscopy and flow cytometry, we demonstrate a switch in potassium channel expression during differentiation of human B cells from naive to memory cells. Naive and IgD⁺CD27⁺ memory B cells express small numbers of the voltage-gated Kv1.3 and the Ca²⁺-activated intermediate-conductance IKCa1 channel when quiescent, and increase IKCa1 expression 45-fold upon activation with no change in Kv1.3 levels. In contrast, quiescent class-switched memory B cells express high levels of Kv1.3 (~2000 channels/cell) and maintain their Kv1.3-high expression after activation. Consistent with their channel phenotypes, proliferation of naive and IgD⁺CD27⁺ memory B cells is suppressed by the specific IKCa1 inhibitor TRAM-34 but not by the potent Kv1.3 blocker Stichodactyla helianthus toxin, whereas the proliferation of class-switched memory B cells is suppressed by Stichodactyla helianthus toxin but not TRAM-34. These changes parallel those reported for T cells. Therefore, specific Kv1.3 and IKCa1 inhibitors may have use in therapeutic manipulation of selective lymphocyte subsets in immunological disorders.


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MS (17, 19), type-1 diabetes (23), and rheumatoid arthritis (24). A minor population of IgD CD27+ B cells exists in most donors, but their functional role has yet to be defined (21).

Mature naive B cells were reported over a decade ago to express Kv and K channels with properties resembling those of Kv1.3 and IKCa1. Studies with nonspecific K+ channel inhibitors suggested a functional role for these channels in B cell mitogenesis (25–29). Because the changes in K+ channel phenotype are functionally important in the T cell lineage, we were interested in determining whether a parallel switch in K+ channel expression accompanies differentiation from naive to memory cells in the B cell lineage. Therefore, we examined the expression and functional role of Kv1.3 and IKCa1 during the differentiation of human B cells from naive to memory cells using whole-cell patch-clamp recording in combination with fluorescence microscopy, flow cytometry, and specific channel blockers. Our studies demonstrate that a switch in K+ channel expression accompanies B cell differentiation that parallels changes seen in the T cell lineage. The plasticity of K+ channel expression in both the B and T cell lineages and its functional ramifications afford promise for specific immunomodulatory actions of K+ channel blockers.

Materials and Methods

Channel blockers

ShK, charybdotoxin, margatoxin, ShK-Dap2, and ShK-F6CA were from Bachem Biosciences (King of Prussia, PA). TRAM-34 and Psora-4 were synthesized as previously described by our group (30, 31). Dendrotoxin-I and dendrotoxin-k were purchased from Sigma-Aldrich (St. Louis, MO).

B cell isolation

Peripheral blood (PB) CD19+ cells were negatively selected from venous blood of healthy volunteers with RosetteSep B cell enrichment mixture (StemCell Technologies, Vancouver, BC, Canada). Human tonsil samples were obtained under an Institutional Review Board-approved protocol. Within 1 h after tonsillec- tomy, mononuclear cells were prepared by slicing the sample into pieces and forcing them through a 70-μm filter to get a single-cell suspension. CD19+ cells were isolated using the StemSep B Cell Negative Isolation System according to the manufacturer’s instructions. Cells isolated from both the blood and the tonsil were found to be 99.2–99.6% CD19+ by flow cytometry (PE-conjugated mouse anti-human CD19 mAb; HB19; BD Pharmingen, San Diego, CA). IgD− cells were positively selected from CD19+ cells with a biotinylated anti-IgD mAb (Ia-2; BD Pharmingen) followed by an anti-biotin tetrameric Ab complex and magnetic beads (StemSep System; StemCell Technologies). From the column flow-through containing IgD− B cells, we positively selected IgD+ CD27+ cells with a custom-made anti-CD27 Ab mixture and magnetic nanobeads according to the manufacturer’s protocol (EasySep System; StemCell Technologies).

B cell culture and activation

CD19+ cells from PB or tonsil were cultured in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM NaHCO3, and 5 mM HEPES (pH 7.4; 300 mOsm). To reduce chloride leak currents, we used a Na+ aspartate external solution containing 160 mM Na+ aspartate, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH 7.4; 300 mOsm). Whole-cell IKCa1 conductances were calculated from the slope of the current-voltage relationship at −80 mV. Kv1.3 and IKCa1 channel numbers per cell were determined by dividing the channel conductance by the single-channel conduc-
tance value for each channel: Kv1.3, 12 ps; IKCa1, 11 ps (14, 35). Cell capacitance, a direct measure of cell surface area, was constantly moni-
tored during recording and noted for each cell, and the surface of each cell was measured by the formula: 1 ps = 100 μm2. To normalize for cell size, we determined Kv1.3 and IKCa1 channel densities for each cell by dividing the channel number per cell by that cell’s surface area.

Statistical differences in channel numbers per cell and channel density per square micrometer between different B cell subsets were determined by one-way ANOVA with a significance level of p < 0.05.

Three-color flow cytometry

Negatively selected CD19+ cells from PB or tonsil were stained for CD19, IgD, CD80, CD86, or CD38 immediately after isolation. Cells were incubated in PBS containing 2% goat serum with a combination of FITC-conjugated mouse anti-human IgD mAb (Ia-6-2), PE-conjugated mouse anti-human CD27 mAb (clone M-T271), and CyChrome-conjugated mouse anti-human CD80 Ab (clone L307.1) or CyChrome-conjugated mouse anti-human CD86 Ab (clone 2331; all BD Pharmingen). A BD Biosciences (San Jose, CA) FACScan with CellQuest software was used to analyze the stained cells.

Immunohistochemistry

Paraffin-embedded sections from human tonsil and spleen (donor >30 years of age) were acquired from the University of California, Irvine, Pathology Department, under an Institutional Review Board-approved protocol. Sections were dewaxed with xylene and rehydrated through an alcohol gradient. Before staining with primary Abs, sections were heated in a microwave for 15 min to retrieve antigenic determinants masked by paraffin embedding. After treatment with 1% H2O2 to inactivate endogenous peroxidase activity and blocking with 5% goat serum in PBS for 1 h, sections were incubated with polyclonal rabbit primary Abs for Kv1.3 (1:500 of 4.1 μg/ml IgG) or IgD (1:500; A0093; DakoCytomation) or CD27 (1:100; H-260; Santa Cruz Bio-technology, Santa Cruz, CA) for 2 h at room temperature. Bound primary Abs were detected with a biotinylated goat anti-rabbit secondary Ab (1 h; 1:1000; Jackson ImmunoResearch, West Grove, PA) followed by a HRP-conjugated avidin complex (Vectorstain Elite ABC kit; Vector Laboratories, Burlingame, CA). After each incubation step, sections were rinsed with PBS three times for 5 min. Peroxidase activity was visualized with 3.3′-diaminobenzidine (DAB substrate kit for peroxidase; Vector Laboratories). Sections were counterstained with hematoxylin (Fisher Scientific, Hampton, NH), dehydrated, and mounted with Permount (Fisher Scientific). The polyclonal rabbit Kv1.3 Ab was previously shown to be specific for Kv1.3 (36).

Electrophysiology

Channel expression was studied in the four B cell subsets defined by expression of IgD and CD27 before or after activation with PMA and ionomycin or with anti-CD40 Ab in the whole-cell mode of the patch-clamp technique with an EPC-9 HEKA amplifier (HEKA Elektronik Dr. Schulze, Lambrecht, Germany). All experiments were conducted at room tempera-
ture, and series resistance compensation was used for Kv currents when they exceeded 2 nA. Cells were stained for IgD and CD27 on ice in com-
plete RPMI with FITC-conjugated mouse anti-human CD27 mAb (M-
T271) and PE-conjugated mouse anti-human IgD mAb (IA-6-2; both BD Pharmingen). Cells were washed, put on poly-l-lysine-coated coverslips, kept in the dark at 4°C for 10–30 min to attach, visualized by fluorescence microscopy, and patch-clamped in the whole-cell configuration. For experiments on CD27 IgA and CD27 IgG class-switched B cells, we stained CD27+ cells with the conjugated mouse anti-human CD27 mAb (M-T271; BD Pharmingen) and FITC-conjugated Fab′(2) of rabbit anti-
human IgA or anti-human IgG (both DakoCytomation, Carpenteria, CA). Kv1.3 currents were elicited by repeated 200-ms pulses from a holding potential of −80 to 40 mV applied every 30 s unless otherwise stated. Kv1.3 currents were recorded in normal Ringer solution with a Ca2+-free pipette solution containing 145 mM KF, 10 mM HEPES, 10 mM KEGTA, and 2 mM MgCl2 (pH 7.2; 300 mOsm). Whole-cell Kv1.3 conductance was calculated from the peak current amplitude at 40 mV.

IKCa1 currents were elicited with voltage ramps from −120 to 40 mV of 200-ms duration applied every 10 s. The pipette solution contained 145 mM K+ aspartate, 2 mM MgCl2, 10 mM HEPES, 10 mM KEGTA, and 8.5 mM CaCl2 (1 μM free Ca2+) (pH 7.2; 290 mOsm). To reduce chloride leak currents, we used a Na+ aspartate external solution containing 160 mM Na+ aspartate, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH 7.4; 300 mOsm). Whole-cell IKCa1 conductances were calculated from the slope of the current-voltage relationship at −80 mV. Kv1.3 and IKCa1 channel numbers per cell were determined by dividing the channel conductance by the single-channel conduc-
tance value for each channel: Kv1.3, 12 ps; IKCa1, 11 ps (14, 35). Cell capacitance, a direct measure of cell surface area, was constantly moni-
tored during recording and noted for each cell, and the surface of each cell was measured by the formula: 1 ps = 100 μm2. To normalize for cell size, we determined Kv1.3 and IKCa1 channel densities for each cell by dividing the channel number per cell by that cell’s surface area.

Statistical differences in channel numbers per cell and channel density per square micrometer between different B cell subsets were determined by one-way ANOVA with a significance level of p < 0.05.

Tonsillar IgD or IgD CD27+ B cells (1 × 107 cells per well) were incubated in complete RPMI medium in 96-well plates in the presence or absence of 10 nM PMA plus 175 nM ionomycin, and with or without ShK (Bachem Biosciences) or TRAM-34 (30). [3H]Thymidine (1 μCi/well) was added after 24 h to the IgD CD27+ (which responded faster than the IgD− cells) and after 36 h to the IgD- cells. ShK and TRAM-34 were chosen for these experiments as Kv1.3 and IKCa1 blockers, because both compounds have demonstrated therapeutic efficacy in animal models of disease (16).
Kv1.3 IN MEMORY B CELLS

Results

K⁺ channel expression pattern of quiescent class-switched memory B cells distinguishes this subset from other B cells

Using the pan-B cell marker CD19, we isolated B lymphocytes from PB of healthy volunteers (>98% purity) and stained them with fluorophore-conjugated Abs specific for IgD (orange, PE) and CD27 (green, FITC). The different B cell subsets were visualized by fluorescence microscopy, and their K⁺ channel expression patterns were determined by whole-cell patch clamp. In the example shown in Fig. 1a, the patch-clamped IgD⁺CD27⁺ memory cell is distinguished from the surrounding naive IgD⁺CD27⁻ cells by its currents in both subsets by ShK. Currents were elicited by 200-ms depolarizing pulses from −80 to 40 mV applied every 1 s. The Kv1.3-specific ShK derivative ShK-F6CA completely inhibits the Kᵥ current in both IgD⁺CD27⁺ (left) and IgD⁺CD27⁻ (right) B cells, whereas the Kv1.1 blocker DTX-I has no effect on the current at 100 nM. The Kv1.3 current was completely blocked by 1 μM ShK-Dap² in this experiment. c, Kv1.3 and IKCa1 currents in IgD⁺CD27⁻ (left) and IgD⁺CD27⁺ (right) cells. The current seen at voltages below −40 mV is carried by IKCa1, whereas the current at more positive voltages is a combination of Kv1.3 (blocked by 1 μM ShK-F6CA) and IKCa1 (blocked by 250 nM TRAM-34). The inset in the right panel shows the current in the IgD⁺CD27⁻ naive cell on a ×15 expanded scale.

[³H]Thymidine incorporation was then determined after a further 12 h. Counts in the presence of blockers were normalized to maximal counts from the same experiment using the following formula: blocker cpm − resting cpm/maximal cpm − resting cpm (counts for IgD⁺ cells were typically −80,000 cpm, and for IgD⁺CD27⁻ cells −10,000 cpm). For anti-CD40 activation, 5 × 10⁴ IgD⁺ or IgD⁺CD27⁻ cells were incubated with 2 × 10⁶ irradiated CD32-transfected K562 cells and 1 μg/ml anti-CD40 mAb for 48 h (IgD⁺CD27⁺) or 96 h (IgD⁺) in round-bottom 96-well plates to allow sufficient contact. [³H]Thymidine was added for the last 12 h of culture (maximal counts for IgD⁺ cells were 35,000 cpm, and for IgD⁺CD27⁻ cells 7,000 cpm).

FIGURE 1. IgD⁺CD27⁺ memory B cells express much larger Kv1.3 currents than naive B cells. a, Transmitted light (left) and fluorescent image (right) of CD1⁹⁺ B cells stained with PE-conjugated IgD and FITC-conjugated CD27 Abs. A IgD⁺CD27⁺ memory B cell is patch-clamped. b, Kv1.3 currents in both IgD⁺CD27⁺ (left) and IgD⁺CD27⁻ naive (right) B cells exhibit the characteristic use-dependence of Kv1.3. Currents were elicited by 200-ms pulses from −80 to +40 mV applied every 1 s. The numbers 1, 2, and 3 above the traces refer to the first, second, and third depolarizing pulse. The inset in the right panel shows the current in the IgD⁺CD27⁻ naive cell on a ×18 expanded scale. c, Normalized peak K⁺ conductance-voltage relationship for the Kv1.3 current in IgD⁺CD27⁺ memory (left) and IgD⁺CD27⁻ naive (right) B cells (n = 3). The line through the points was fitted with the Boltzmann equation. d, Blockade of Kv1.3 currents in both subsets by ShK. currents were elicited by 200-ms depolarizing pulses from −80 to 40 mV applied every 30 s. e, The Kv1.3-specific ShK derivative ShK-F6CA completely inhibits the Kv1.3 current in both IgD⁺CD27⁺ (left) and IgD⁺CD27⁻ (right) B cells, whereas the Kv1.1 blocker DTX-I has no effect on the current at 100 nM. f, Dose-dependent block of Kv1.3 currents in both subsets by Psora-4, the most potent small-molecule inhibitor of Kv1.3. All experiments shown in b-f were conducted with a Ca²⁺-free KF-based patch pipette solution.
The amplitude of both the Kv1.3 and the fiT cells and cloned IKCa1 are from Refs. 14, 30, and 86. Figs. 1 and 2; Table I). The amplitude of both the Kv1.3 and the calcium-activated (IKCa1) K+ currents with the characteristic fingerprints of Kv1.3 and IKCa1 channels (Figs. 1 and 2; Table I). The amplitude of both the Kv1.3 and the IKCa1 current was substantially larger in the IgD+CD27+ class-switched memory B cells than in IgD−CD27− naive B cells (Figs. 1 and 2). Staining with the anti-IgD and the anti-CD27 Abs did not alter the properties or the expression of the Kv1.3 and IKCa1 currents (data not shown).

Kv currents in both B cell subsets exhibited use-dependence, a property characteristic of Kv1.3, in which rapid repetitive depolarizing pulses cause a progressive decrease in the Kv current amplitude due to channel trapping in the inactivated state (Fig. 1b). Their half-activation voltage and inactivation time-constant were also similar to Kv1.3 (Fig. 1c; Table I). The polypeptides ShK (Fig. 1d), ShK-Dap (22), charybdotoxin, and margatoxin blocked the B cell Kv currents with the same potencies as Kv1.3 (Table I). ShK-F6CA, an analog of ShK (37) that exhibits ~80-fold selectivity for Kv1.3 over closely related Kv family channels, and Psor-4 (31), the most potent small molecule inhibitor of Kv1.3, also blocked the Kc currents at concentrations that block Kv1.3 (Fig. 1, e and f; Table I). Dendrotoxin-1 and dendrotoxin-κ, two polypeptides that inhibit the Kv1.1 channel that has been reported in mouse thymocytes (38) and mouse T cells (39, 40) but not in human T cells (6), had no effect on the Kc currents in the B cell subsets (Fig. 1e; Table I). Taken together, these results show that the biophysical and pharmacological properties of the Kc channel in human B cells are identical to those of Kv1.3, and suggest that Kv1.3 is the only Kc channel expressed in human B cells (14–16, 35).

We also observed Kc currents in both B cell subsets when recording with a pipette solution containing 1 mM free calcium. In the ramp protocol from an IgD−CD27− memory B cell a calcium-dependent, voltage-independent, weakly inwardly rectifying Kc current is seen that reverses at ~80 mV (Fig. 2a). This current was blocked by the IKCa1-specific inhibitor TRAM-34 (Fig. 2b) and by charybdotoxin with potencies identical to those of the cloned IKCa1 channel (Table I). Together, these results strongly argue that the IKCa1 channel underlies the Kc currents in naive and class-switched human B cells. Furthermore, the complete inhibition of all K+ current by the combination of 1 nM ShK-F6CA and 250 nM TRAM-34 (Fig. 2, c and d) demonstrated that Kv1.3 and IKCa1 are the only K+ channels present in circulating human B cells.

The four B cell subsets defined by CD27 and IgD expression are shown in the flow cytometry profile in Fig. 3a. The relative proportions of each subset varied in different individuals: naive IgD−CD27− cells (45–73% in five donors), IgD+CD27+ memory cells (3–21%), class-switched IgD+CD27+ memory cells (4–20%), and IgD−CD27− cells (2–11%). The activation markers CD86 and CD38 were not expressed or expressed only at very low levels in all four subsets (data not shown), suggesting that these cells were quiescent. The majority (88–95%) of class-switched memory cells expressed cell surface CD80 (CD80 = B7.1 = ligand for CD28), whereas only 20–40% of IgD+CD27+ memory cells expressed this marker and naive B cells were CD80 negative (Fig. 3b). Identical results were obtained with tonsillar B cells.

Representative Kv1.3 and IKCa1 currents of the four major B cell subsets are shown next to the respective FACS quadrants in Fig. 3a. The properties of the Kv1.3 and Kc currents were identical in the four B cell subsets. The amplitude of the Kv1.3 and IKCa1 currents in quiescent class-switched B cells was significantly larger than in the other three B cell subsets. We determined channel numbers per cell by dividing the whole-cell conductance for each channel in each cell with the corresponding single-channel conductance values. The Kv1.3 and IKCa1 channel numbers per cell in the four quiescent B cell subsets are shown in Fig. 4a. Naive B cells expressed small numbers of Kv1.3 (~100/cell) and IKCa1 (~5/cell) channels. IgD−CD27− memory B cells expressed more Kv1.3 channels (~250/cell) than naive cells but the same number of IKCa1 channels (~7/cell). IgD+CD27+ cells also expressed small numbers of Kv1.3 (~70/cell) and IKCa1 (~18/cell) channels. In contrast, class-switched IgD+CD27+ cells expressed 10–20 times more Kv1.3 channels (~2400/cell; p < 0.00001 for all three cases) and 4–8 times more IKCa1 channels (~60/cell; p < 0.00001 (naive and IgD−CD27−); p = 0.00234 (IgD+CD27+)) than the other three subsets. This channel expression pattern has not been seen before in any quiescent lymphoid subset. The corresponding B cell subsets in the human tonsil exhibited K+ channel expression patterns identical with their PB counterparts (see Fig. 4a). Because class-switched memory B cells are significantly larger than the other three subsets, we normalized channel expression levels for cell size by calculating the channel density per square micrometer of surface area. Class-switched B

Table 1. Comparison of the biophysical and pharmacological properties of the Kv and Kc currents in human B cells with the Kv and Kc currents in human T cells and the cloned Kv1.3 and IKCa1 channels*

<table>
<thead>
<tr>
<th>Kv</th>
<th>IgD−CD27−</th>
<th>IgD−CD27+</th>
<th>Kv1.3 Human T Cells</th>
<th>Cloned Kv1.3</th>
<th>IKCa1 Human T Cells</th>
<th>Cloned IKCa1</th>
</tr>
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<tr>
<td>V1/2</td>
<td>−32 mV</td>
<td>−31 mV</td>
<td>−36 mV</td>
<td>−35 mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>τr</td>
<td>240 ± 58 ms</td>
<td>260 ± 69 ms</td>
<td>178 ms</td>
<td>250 ± 51 ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShK</td>
<td>11 ± 2 μM</td>
<td>10 ± 1 μM</td>
<td>10 ± 1 μM</td>
<td>11 ± 2 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShK-Dap</td>
<td>58 ± 12 μM</td>
<td>55 ± 9 μM</td>
<td>52 ± 10 μM</td>
<td>25 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charybdotoxin</td>
<td>3.0 ± 0.5 nM</td>
<td>3.2 ± 0.5 nM</td>
<td>2.5 nM</td>
<td>2.6 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Margatoxin</td>
<td>115 ± 8 μM</td>
<td>108 ± 14 μM</td>
<td>110 ± 16 μM</td>
<td>100 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShK-F6CA</td>
<td>39 ± 8 μM</td>
<td>42 ± 10 μM</td>
<td>ND</td>
<td>48 ± 4 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psor-4</td>
<td>3.2 ± 0.5 nM</td>
<td>2.7 ± 0.5 nM</td>
<td>3.0 ± 0.3 nM</td>
<td>2.9 ± 0.3 nM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendrotoxin-1</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendrotoxin-κ</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
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<td></td>
</tr>
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</table>

* Kv1.3: Compounds were tested at least three times at three to five concentrations, and Kc values were determined by fitting the resulting inhibition of peak current at 40 mV with the Hill equation. Dendrotoxin-1 and its Kv1.1-selective derivative dendrotoxin-κ showed no effect at 100 nM. Values for human T cells and Kv1.3 are from Refs. 2, 14, 15, 31, 35, 37, 84, and 85. V1/2, Half-activation voltage; τr, inactivation time constant. IKCa1: Kc values were determined by fitting the reduction of slope conductance at ~80 mV to the Hill equation. The measurements on IgD−CD27− B cells were performed after activation because the Kc currents are much larger (see Fig. 4a). Values for human T cells and cloned IKCa1 are from Refs. 14, 30, and 86.
cells had 6- to 21-fold higher Kv1.3 ($p < 0.00001$ in all three cases) and 2- to 8-fold higher IKCa1 channel densities ($p = 0.00012$–0.05663) than the other three subsets (Fig. 4a).

Class-switched IgD+CD27+ memory cells express other Ig isotypes on the cell surface in place of IgD (20, 22). Therefore, patch-clamp recording was done on IgA+CD27+ and IgG+CD27+ cells visualized by fluorescence microscopy. IgA+CD27+ (Fig. 4b, left) and IgG+CD27+ (middle) cells expressed large Kv1.3 currents that exhibited use-dependence. IgG+ cells expressed slightly higher Kv1.3 channel numbers per cell than IgA+ cells ($p < 0.05$). Both class-switched subsets expressed 10- to 20-fold more Kv1.3 channels than naive or IgD+CD27+ cells (Fig. 4b, right).

**FIGURE 3.** K⁺ channel expression in B cells subsets. a, Flow cytometry profile showing IgD and CD27 expression in quiescent CD19+ B cells. Four subsets are distinguished: IgD+CD27+ (naive), IgD+CD27+ (class-switched memory B cell), and IgD+CD27+. Representative Kv1.3 and IKCa1 currents are shown next to each FACS quadrant. Kv1.3 currents were recorded with depolarizing steps from −80 to 40 mV every 30 s with KF-based pipette solution. IKCa1 currents were elicited by 200-ms ramp pulses from −120 to 40 mV with 1 µM free Ca²⁺ in the pipette solution and 1 nM specific Kv1.3 blocker ShK-Dap (22, 84) in the bath. b, Flow cytometry profile showing CD80 expression on naive IgD+CD27+ (left), IgD+CD27+ (middle), and class-switched IgD+CD27+ (right) B cells.
In summary, differentiation of human B cells from a naive to a class-switched memory B cell stage is accompanied by a change in K^+ channel expression. Class-switched B cells express significantly higher numbers of Kv1.3 and IKCa1 channels than the other three B cell subsets. Our flow cytometry data show that the majority of class-switched B cells express the activation marker CD80, the ligand for CD28. Interestingly, the CD27^+CD80^+ subset (class-switched B cells based on our results) has been reported to effectively present Ag to CD4^+ T cells without requiring pre-activation, activate at lower thresholds, and differentiate rapidly into cells that secrete large amounts of class-switched Abs (41), and has also been implicated in autoimmune pathophysiology (42–45). The Kv1.3^high pattern of these cells may position them for rapid activation.

**FIGURE 4.** Numbers of K^+ channels expressed per cell in resting and activated cells of the four B cell subsets. **a,** Scatterplot of Kv1.3 and IKCa1 channel numbers per cell in the four B cell subsets from PB in the resting state (○, △, □, ◊) and after activation (●, ▲, ■, ♦). The bar shows the mean of the respective subset. Mean channel numbers per cell (±SEM), mean cell capacitance (a measure of cell size), and mean Kv1.3 and IKCa1 channel densities are shown in a table below the figure. Similar results were obtained with tonsillar B cells: naive resting (63 ± 4 Kv1.3 channels, n = 10; 3 ± 1 IKCa1 channels, n = 6); naive activated (108 ± 10 Kv1.3, n = 15; 742 ± 90 IKCa1, n = 14); IgD^+CD27^+ resting (245 ± 26 Kv1.3, n = 8; 8 ± 1.2 IKCa1, n = 5); IgD^+CD27^+ activated (213 ± 20 Kv1.3, n = 13; 880 ± 92 IKCa1, n = 12); class-switched IgD^+CD27^+ resting (1817 ± 124 Kv1.3, n = 11; 47 ± 13 IKCa1, n = 7); and class-switched IgD^+CD27^+ activated (2285 ± 232 Kv1.3, n = 14; 80 ± 11 IKCa1, n = 8). A one-way ANOVA test revealed that the Kv1.3 and IKCa1 expression levels were not significantly different between PB or tonsillar B cells from each subset. **b,** Kv1.3 currents (left and middle) and Kv1.3 channel numbers per cell (right) in class-switched CD27^+IgA^+ (1648 ± 364 Kv1.3, 3.8 ± 0.2 pF, n = 12) and CD27^+IgG^+ (1966 ± 234 Kv1.3, 3.7 ± 0.2 pF, n = 12) B cells.
IKCa1 is up-regulated in naive and IgD+CD27+ B cells following activation, whereas class-switched memory B cells retain their Kv1.3^{high} expression following activation

We examined the effect of mitogenic activation on channel expression in the four human B cell subsets. PB CD19+ B lymphocytes were stimulated for 30–96 h with anti-CD40 Ab (presented by CD32-transfected K562 cells (34)) to mimic T cell-mediated activation through CD40 (33, 46), or with a combination of PMA plus ionomycin (32). Cells were immunostained for IgD and CD27, and then visualized by fluorescence microscopy and patch clamped. Analysis of multiple cells showed that naive, IgD+CD27+ and IgD−CD27− B cells, up-regulated IKCa1 expression following activation, whereas Kv1.3 levels did not change (Fig. 4a). When normalized for cell size, IKCa1 density in activated cells of these three subsets was ~45-fold higher (p < 0.000001) than in the resting state, whereas Kv1.3 density was 4-fold lower (p = 0.00003). In contrast, class-switched memory B cells augmented Kv1.3 levels following activation, but this increase was proportionate to the change in size and did not alter channel density (Fig. 4a). IKCa1 density in these cells did not change with activation. Similar results were obtained with tonsillar B cells (Fig. 4a). Thus, activation enhances IKCa1 density and reduces Kv1.3 density in naive, IgD+CD27+ and IgD−CD27− B cells, but has no significant effect on channel density in class-switched IgD−CD27+ memory B cells, which retain their Kv1.3^{high} pattern.

Kv1.3^{high} B cells reside in memory compartments of human lymphoid tissues

The number of Kv1.3 channels (~2000 channels/cell) in quiescent class-switched memory B cells should be sufficiently high to detect Kv1.3 protein within the B cell compartments of human secondary lymphoid tissues by immunostaining with Kv1.3-specific Abs. To determine the regional distribution of K+ channels in intact tissue samples and to compare it with our electrophysiological data, we stained paraffin sections of human tonsil and spleen with a polyclonal Ab that has been previously demonstrated to be specific for Kv1.3 (36). In the tonsillar section shown in Fig. 5a, a ring of IgD+ cells is seen in the mantle of the B cell follicles surrounding the germinal center (GC). Robust Kv1.3 staining was detected within the GC where class-switched memory B cells are found (Fig. 5b) but not in the mantle where IgD− B cells reside. The expression pattern in the GC varied—in some cases, Kv1.3+ cells were dispersed throughout (Fig. 5b), whereas in others, they were clustered at one edge of the GC (d). Additional Kv1.3 staining was seen outside the GC (Fig. 5c) in a region that has been described as marginal zone (MZ)-like, because it contains IgD− class-switched memory B cells (47, 48). Serial sections stained for Kv1.3 (Fig. 5d) and CD27 (e) confirmed that these markers were expressed on the same population of cells in the GC and the MZ-like area. No significant Kv1.3 staining was seen in the T cell zone (data not shown) consistent with the low Kv1.3 expression levels of resting naive, T_{EM} and T_{EM} cells (15). In the human spleen, Kv1.3 and CD27 staining was detected in the MZ (data not shown) where CD27+ B cells are located (49, 50). No staining was observed with rabbit control IgG. In summary, Kv1.3^{high} cells are found in the GC and MZ of the tonsil and spleen where class-switched memory B cells reside or traffic through.

IKCa1 blockade suppresses proliferation of naive B cells and IgD−CD27+ memory B cells, whereas Kv1.3 blockade suppresses proliferation of class-switched memory B cells

The availability of highly specific Kv1.3 and IKCa1 inhibitors and the subset-specific patterns of Kv1.3 and IKCa1 expression raise the possibility of using IKCa1-specific inhibitors to suppress the proliferation of naive and IgD−CD27+ memory B cells and Kv1.3

![FIGURE 5](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org) Localization of IgD+, Kv1.3+ and CD27+ cells in the human tonsil. a, IgD+ cells outline the mantle (M) of the B cell follicles (×40). b and c, Positive Kv1.3 staining is seen in the GC (b) and the adjacent region that is consistent with the MZ (c) (both ×200). d and e, Kv1.3 (d) and CD27 (e) stain the same areas in serial sections from human tonsil (×100). All sections apart from the one shown in a are counterstained with hematoxylin.
blocks to suppress class-switched Kv1.3\textsuperscript{high} memory B cells. To test this idea, human tonsillar CD19\textsuperscript{+} B cells were isolated and then further separated into two subsets, an IgD\textsuperscript{+} subset containing both naive and IgD\textsuperscript{CD27\textsuperscript{+}} cells, and a second subset containing class-switched IgD\textsuperscript{CD27\textsuperscript{+}} memory B cells. Both subsets were activated with anti-CD40 Ab (33, 46) or with a combination of PMA and ionomycin (32, 33, 51–56) in the presence or absence of the IKCa1 blocker TRAM-34 or the Kv1.3 blocker ShK, and [\textsuperscript{3}H]thymidine incorporation was measured 24–72 h later. In keeping with our expectation, TRAM-34 suppressed the proliferation of the IgD\textsuperscript{+} subset (Fig. 6) with EC\textsubscript{50} values of 200 nM (anti-CD40 Ab) and 100 nM (PMA plus ionomycin), and ShK had no effect at a concentration (10 nM) that completely blocks Kv1.3 channels. At a 10-fold higher ShK concentration (100 nM) that also affects IKCa1 channels, 40–50% suppression was observed. Also, as anticipated, ShK suppressed the proliferation of class-switched Kv1.3\textsuperscript{high} IgD\textsuperscript{CD27\textsuperscript{+}} memory B cells with EC\textsubscript{50} values of 1 nM (anti-CD40 Ab) and 4 nM (PMA plus ionomycin), concentrations that selectively block Kv1.3, whereas TRAM-34 was ineffective (EC\textsubscript{50} > 1 \textmu M) (Fig. 6). Similar results were obtained with Pso-ra-4, the most potent small molecule inhibitor of Kv1.3 (31). Pso-ra-4 suppressed the proliferation of IgD\textsuperscript{CD27\textsuperscript{+}} memory B cells with an EC\textsubscript{50} of 250 nM (data not shown) but had no effect on the proliferation of IgD\textsuperscript{+} B cells at concentrations up to 1 \textmu M. These results indicate that Kv1.3 blockers preferentially suppress the proliferation of class-switched memory B cells, whereas IKCa1 blockers suppress the proliferation of naive and IgD\textsuperscript{CD27\textsuperscript{+}} B cells.

**Discussion**

Using whole-cell patch-clamp recording in conjunction with fluorescence microscopy, we demonstrate a switch in K\textsuperscript{+} channel dominance from IKCa1 to Kv1.3 during differentiation of naive and IgD\textsuperscript{CD27\textsuperscript{+}} B cells into class-switched memory B cells. As shown in Fig. 7, naive and IgD\textsuperscript{CD27\textsuperscript{+}} memory B cells up-regulate IKCa1 expression during activation, and their proliferation is suppressed by the specific IKCa1 inhibitor TRAM-34 (6) but not by Kv1.3 blockade. In contrast, class-switched IgD\textsuperscript{CD27\textsuperscript{+}} memory B cells express high levels of Kv1.3 channels in the resting state, a pattern not seen previously in any lymphoid subset. The Kv1.3 inhibitor ShK suppressed mitogen-driven proliferation of this subset, whereas the IKCa1 inhibitor TRAM-34 was much less effective (Fig. 7). These Kv1.3\textsuperscript{high} class-switched memory cells reside in the GC and MZs of the human tonsil and spleen. The functional dominance of IKCa1 in naive and IgD\textsuperscript{CD27\textsuperscript{+}} B cells, and Kv1.3 in class-switched memory B cells offers a powerful way to manipulate the activity of these subsets with specific IKCa1 and Kv1.3 inhibitors.

The changes in K\textsuperscript{+} channel expression observed in the B cell lineage parallel those reported to be seen in the T cell lineage (Fig. 7b). Naive T cells and T\textsubscript{EM} cells start with 300 Kv1.3 and 10 IKCa1 channels/cell, and up-regulate IKCa1 expression to ~500–600/cell with little change in Kv1.3 numbers (15, 37). T\textsubscript{EM} cells, in contrast, up-regulate Kv1.3 upon activation with no change in IKCa1 expression. Thus, naive and early memory (IgD\textsuperscript{CD27\textsuperscript{+}}; T\textsubscript{EM}) cells of both lineages up-regulate IKCa1 during activation, whereas late memory (class-switched B cells, T\textsubscript{EM}) cells of both lineages up-regulate Kv1.3 (Fig. 7).

Differences in the ratio of IKCa1 and Kv1.3 numbers may contribute to differences in Ca\textsuperscript{2+} signaling patterns and thereby influence the function of distinct T and B cell subsets. The shape and the nature of the Ca\textsuperscript{2+} signal regulate gene expression in response to antigenic stimulation (57, 58). For example, lymphocytes in which IKCa1 predominates (e.g., activated naive and early memory cells) have been reported to have an enhanced tendency to exhibit oscillatory Ca\textsuperscript{2+} signals in response to stimulation, presumably because calcium entry is tightly coupled to the opening of IKCa1 channels (13, 59, 60). Kv1.3 might promote trafficking of lymphocytes to inflamed tissues via its physical and functional coupling to \beta_1 integrins and its reported role in cell adhesion and migration (61). As suggested by two recent papers on Kv1.3-transfected Jurkat and human CTLs, Kv1.3 may also play an important role in formation of the immunological synapse (62) and in the interaction of CD8\textsuperscript{+} cells with their targets (63).

The differential channel expression patterns in naive and early memory cells vs late memory cells in both lineages are responsible for their differential sensitivities to Kv1.3 and IKCa1 blockers in proliferation assays (Fig. 7b). Because Kv1.3 channels predominate in resting naive T cells and T\textsubscript{EM} cells, these subsets are initially sensitive to Kv1.3 blockade but escape inhibition within 48 h through up-regulation of IKCa1 (6, 14, 15). During reactivation, TRAM-34 suppresses proliferation of these cells, whereas Kv1.3 blockers are ineffective. In contrast, the proliferation of naive and early memory B cells is suppressed by the IKCa1-specific inhibitor TRAM-34 and not Kv1.3, presumably because these cells start with small Kv1.3 currents, up-regulate IKCa1 rapidly, and take longer to activate than T cells. In the late memory cell pool, T\textsubscript{EM} cells are highly sensitive to Kv1.3 but not IKCa1 blockers because they contain more Kv1.3 than IKCa1 channels in the resting state and augment this difference further during activation (Fig. 7b). Kv1.3 blockers also suppress mitogen-driven proliferation of class-switched memory B cells but with 10-fold lower potency than T\textsubscript{EM} cells (Fig. 7b), possibly because memory B cells start...
with 5- to 10-fold higher numbers of Kv1.3 (≈2000/cell) channels in the quiescent state than T_{EM} cells (200–400/cell).

Because IKCa1 is the functionally dominant K^+ channel in naïve and early memory B and T cells, IKCa1 blockers might have use in suppressing acute immune reactions, for example, during graft rejection and during the acute phases of autoimmune diseases. Clotrimazole, an IKCa1 blocker, was reported to ameliorate rheumatoid arthritis in patients, but was abandoned due to adverse effects resulting from blockade of cytochrome P450 enzymes (64, 65).

The newer IKCa1-specific blockers TRAM-34, ICA-17043, and 4-phenyl-4H-pyran (66–68), which lack this activity, should be evaluated for therapeutic use in autoimmune disorders. Furthermore, cyclosporin A, an immunosuppressant widely used to prevent graft rejection, synergizes with TRAM-34 in T cell proliferation assays.
(30), and combining these compounds could reduce the toxicity that complicates cyclosporin A therapy.

Earlier studies showed that the Kv1.3 blockers margatoxin, correolide, and kaliotoxin suppressed delayed-type hypersensitivity (DTH) in miniswine and rats (5, 69, 70). Based on these results, investigators in the field (5, 71) concluded that Kv1.3 blockers inhibited the primary immune response and caused generalized immunosuppression. However, our in vitro results, presented both in this paper and in earlier reports (15, 16), suggest that Kv1.3 blockers preferentially target late memory cells of both lineages (T_{EM} and class-switched memory B cells), whereas naive and early memory cells, although initially sensitive to Kv1.3 block, up-regulate IKCa1 and escape Kv1.3 inhibition. How might one reconcile these differing views? Recent evidence indicates that essentially all of the T cells isolated from DTH skin in humans exhibit the T_{EM} memory phenotype (72), and T_{EM} cells have the propensity to traffic to the skin (73). It is not surprising then that Kv1.3 blockers effectively inhibited DTH in miniswine via preferential suppression of T_{EM} cells. Specific Kv1.3 blockers might therefore constitute a new class of late memory-specific immunomodulators.

There is an increasing body of evidence implicating late memory cells in the pathogenesis of MS, type-1 diabetes mellitus, rheumatoid arthritis, psoriasis, Crohn’s disease, and chronic graft-vs-host disease (15–17, 74–77). Ag-specific targeting of pathogenic autoreactive T and B cells is the most desirable therapeutic strategy for autoimmune diseases, but Ag-specific strategies may fail due to the phenomenon of epitope spreading. A Kv1.3-based therapeutic approach that targets late memory cells and spares the bulk of the adaptive immune response mediated by naive and early memory cells that depend on IKCa1 channels might be beneficial under these circumstances. For example, channel-based suppression of autoantigen-specific memory B cells that have been reported to contribute to epitope spreading (78), and effectively function as APCs through enhanced autoantigen capture via their Ag-specific membrane-bound Ig (41, 76, 79, 80), may have therapeutic value in autoimmune disorders. In proof-of-concept studies in rats, Kv1.3 blockers have been shown to ameliorate adoptive experimental autoimmune encephalomyelitis induced by myelin-specific memory T cells (16, 70), a model for MS, and to prevent inflammatory bone resorption in experimental periodontal disease caused mainly by memory cells (81). Several small-molecule Kv1.3 inhibitors with nanomolar potency have been developed over the last decade (WIN-17317, CP-339818, U.K.-78,282, correolide, and kaliotoxin suppressed delayed-type hypersensitivity in proliferating B lymphocytes depends upon the mode of activation. J. Clin. Immunol. 21:204.


