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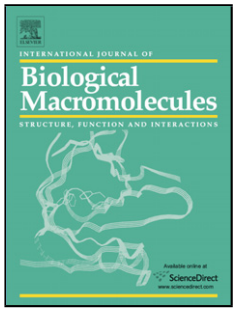

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Dromedary immune response and specific Kv2.1 antibody generation using a specific immunization approach

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ABSTRACT

Voltage-gated potassium (Kv) channels form cells repolarizing power and are commonly expressed in excitable cells. In non-excitable cells, Kv channels such as Kv2.1 are involved in cell differentiation and growth. Due to the involvement of Kv2.1 in several physiological processes, these channels are promising therapeutic targets. To develop Kv2.1 specific antibody-based channel modulators, we applied a novel approach and immunized a dromedary with heterologous *Ltk*- cells that overexpress the mouse Kv2.1 channel instead of immunizing with channel protein fragments. The advantage of this approach is that the channel is presented in its native tetrameric configuration. Using a Cell-ELISA, we demonstrated the ability of the immune serum to detect Kv2.1 channels on the surface of cells that express the channel. Then, using a Patch Clamp electrophysiology assay we explored the capability of the dromedary serum in modulating Kv2.1 currents. Cells that were incubated for 3 hours with serum taken at Day 51 from the start of the immunization displayed a statistically significant 2-fold reduction in current density compared to control conditions as well as cells incubated with serum from Day 0. Here we show that an immunization approach with cells overexpressing the Kv2.1 channel yields immune serum with Kv2.1 specific antibodies.

Keywords: Kv specific serum; immune response; Cell-ELISA; Patch Clamp

1. Introduction

Voltage-gated potassium (Kv) channels are known to create the repolarizing power of excitable cells that terminates the action potential. The Kv2.1 channel is a mammalian orthologue of the shab-family of Kv channels and underlies one of the predominant delayed rectifier K^+ currents in mammalian central nervous system neurons[1]. Besides their role in cell excitability, Kv2.1 channels are also involved in regulating insulin secretion in β-cells and in the differentiation and growth of non-excitable cells [2]. Accordingly, Kv2.1 channels

have been reported to be overexpressed in at least uterine [3] and gastric tumorcells [4, 5]. Several reports indicate that the inhibition of these K^+ currents suppresses the proliferation of cancer-type cells [3-5]. Given the widespread role of Kv2.1 channels in multiple physiological processes, they are considered to be important therapeutic targets for treatment of neurological and cardiovascular disorders, type 2 diabetes and suppression of cancers. Several Kv2.1 selective peptide blockers have been isolated from animal venoms. In particular, Hanatoxin 1 and Hanatoxin 2, isolated from the venom of Chilean *Tarantula* [6], and Ssmtx-1 isolated from the venom of a centipede [7] . However, because of their venom origin, toxins could have adverse effects and their safe therapeutic application must be demonstrated [8]. Therefore, it is more interesting to isolate a non-toxin Kv2.1 modulator, which is able to reduce the K^+ currents. Antibodies that inhibit the channel from the intracellular side have already been reported [9, 10]. To the best of our knowledge, antibodies operating via extracellular regions and modulating the Kv2.1 channels have never been described.

Several studies have highlighted the potential of ion channel specific immunoglobulins to treat a variety of diseases [11-13]. Immunoglobulins or antibody molecules are increasingly being developed for the treatment of tumors. They may represent a novel tool that can be exploited for designing by genetic engineering new Kv2.1 channel modulators. They also represent an interesting tool for immunostaining and biomarker identification. To develop antibody-based modulators of membrane proteins, we questioned whether immunizing dromedary with cells that express the mouse Kv2.1 channel protein is sufficient to induce an immune response and most importantly yields a serum with polyclonal antibodies that recognize and target the Kv2.1 channel. To address this question we developed *Ltk*$^-$ cell lines that stably overexpress the Kv2.1 channel (Fig. 1). Dromedary serum contains, in addition to conventional heterotetrameric camelid IgG1, heavy chain immunoglobulins devoid of both

light chains and conserved CHI domains (i.e. IgG2a and IgG3) [14]. Thus, the heavy chain-only antibodies recognize their cognate antigen by virtue of one single variable domain (instead of paired variable domains). This special antigen binding site prefers to associate with epitopes that are not antigenic for classical antibodies. This unique characteristic could be an advantage in differentially modulating the Kv2.1 channel via extracellular epitopes of the channel.

Herein, we have successfully developed a specific dromedary humoral immune response to Kv2.1 channels as demonstrated using an enzyme-linked immunosorbent assay on cells (Cell-ELISA). Furthermore, the serum of Day 51, which is the serum taken 51 days after onset of the immunization, was able to inhibit the Kv2.1 currents as evaluated by a patch-clamp assay. These results demonstrate that an immunization approach with *Ltk*$^-$cells overexpressing the Kv2.1 channel yields polyclonal antibodies that are able to recognize and to modulate Kv2.1 channels through partial inhibition of the current amplitude without substantial change in the kinetics.

2. Materials and methods

3.1 Cell lines and cell culture

Ltk$^-$cells were cultured in DMEM medium supplemented with 10% horse serum and 1% penicillin/streptomycin under a 5% CO_2 atmosphere at 37°C. The *Ltk*$^-$ cells were stably transfected with the mouse *Kv2.1* channel gene and were cultured in DMEM medium supplemented with 10% horse serum, 1% penicillin/streptomycin and 0.25 mg/ml G418. Both, low and high mouse Kv2.1 expressing *Ltk*$^-$cell lines were constructed using a similar approach as described previously. Subconfluent cultures were incubated with 2 µM dexamethasone for 24 hours to induce Kv2.1 channel expression and the harvested cells were used to immunize and for electrophysiological recordings [15].

3.2 Dromedary immunization

One, three years old and non-pubertal, male camel (*Camelusdromedarius*) weighting 210 kg was provided by the local veterinary at Tantana, Sousse. After a period of quarantine, the dromedary was immunized with *Ltk*$^-$cells expressing Kv2.1 channels, using an optimized protocol of immunization approved by the Comitéd'EthiqueBiomedicale, Institut Pasteur Tunis (Agreement number *CEBM 2015/12/I/LR11IPT08/V0*) and in accordance with the European Directive 2010/63/eu. Preparations of approximately 10^8 viable *Ltk*$^-$cells expressing Kv2.1 channel were mixed with complete (first injection) or incomplete Freund's adjuvant (subsequent boosts) and injected, subcutaneously. The dromedary received successively four doses at days 0, 7, 21 and 51. Blood (5 ml) was collected before the first injection (i.e. Day 0) and during the course of the immunization, just before each injection. The sera were collected and stored at -80°C until further use.

3.3 Cell-ELISA for monitoring the immune response

Cell-ELISA was used to assess the specific titer of the immune response. The 96 wells of maxisorb plates (NUNC) were seeded with *Ltk*- cells overexpressing the Kv2.1 channels or non-transformed *Ltk*$^-$ blank cells (referred to as blank) at roughly 10,000 cells per well and cultured overnight. Overexpression of Kv2.1 channels was induced in the *Ltk*$^-$cells by 2 µM Dexamethasone. After 24 hours induction, cells were gently washed with PBS and subsequently fixed with 1% (w/v) Glutaraldehyde in PBS for 30 minutes at 37°C. Unfixed cells were removed by washing 3 times with PBS. Non-specific binding was prevented by blocking residual protein adsorption sites with 2% Bovine Serum Albumin in PBS for 1 hour at 37°C. Wells were washed three times with PBS and pre-immune and immune sera were added in 2% PBS at the adequate dilution (1:5000). All further incubations were carried out for 1 hour at 37°C and after each incubation, plates were washed ten times with PBS. Bound antibodies were detected by incubating with a rabbit anti-dromedary IgG antiserum (diluted

1:8000 in PBS) and subsequently Goat anti-rabbit IgG Peroxidase conjugate (Sigma). Substrate used was o-Phenylenediamine (Sigma) and the reaction was stopped using 50µl 2M H_2SO_4. The optical density was measured at 492 nm. The assay was performed in triplicate following the same conditions.

3.4 Determination of seeded Ltk$^-$ cells overexpressing Kv2.1 channels

Five 96 wells of maxisorb plates (NUNC) were seeded for each *Ltk*$^-$cells overexpressing Kv2.1 channels and *Ltk*$^-$ cells at roughly 10,000 cells per well and cultured overnight before being induced with2 µM Dexamethasone. After 24 hours, cells were gently washed with PBS, fixed with 1% (w/v) Glutaraldehyde in PBS for 30 minutes at 37°C and unfixed cells were removed by washing 3 times with PBS. 50 µl of crystal violet was added in each well and incubated for 1 hour at 37°C. The plate was then washed with water (at least 30 times). When the wells look clean (absence of blue stain), 50 µl of 1% Sodium Dodecyl Sulfate was added in each well and incubated for 10 minutes at 37°C. The optical density was recorded at 560 nm.

3.5 Electrophysiological recordings

Whole-cell ionic current measurements were performedat room temperature (20 to 23°C) using an Axopatch-200B amplifier. After passing a 5 KHz Bessel low-pass filter, the recordings were digitized at 10 kHz sampling rate with a Digidata-1200A acquisition system (Molecular Devices, Sunnyvale, CA, USA). Command voltages and data storage were controlled with pClamp10 software. Patch pipettes were pulled from 1.2 mm quick-fill borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) with a P-2000 puller (Sutter Instrument Co., Novato, CA, USA) and afterwards heat-polished. The cells were constantly superfused with external bath solution that contained 130 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.35 with NaOH and 10 mM Glucose. The patch pipettes were filled with internal solution containing 110 mM KCl, 5

mM K_4BAPTA, 5 mM K_2ATP, 1 mM $MgCl_2$, 10 mM HEPES adjusted to pH 7.2 with KOH. Junction potentials were zeroed with the filled pipette in the bath solution and experiments were excluded from analysis if the voltage error estimate exceeded 5 mV after series resistance compensation.

For testing the effects of immune sera on Kv2.1 currents, 40 µl of serum (collected at Day 0 or at Day 51) was added to 2 ml medium (2% v/v) before performing the patch clamp experiments and the steady-state current amplitude at +40 mV was recorded and normalized to the cell capacitance.

3.6 Ionic current data analysis

To elicit current activation, depolarizing voltage pulses were applied starting from a −80 mV holding potential. The interpulse interval was 20 s to prevent accumulation of slow inactivation. Current recordings were analyzed with Clampfit10.2 software and graphs were built using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). The conductance versus voltage (GV) curves were fitted with a Boltzmann equation: $y = 1/\{1 + \exp[-(V - V_{1/2})/k]$, where V represents the applied voltage, $V_{1/2}$ the midpoint potential at which half of the channels have opened or inactivated, and k the slope factor. The kinetics of channel activation (opening)and deactivation (closure) were determined by fitting the rise in current activation or decay during deactivation with a single exponential function. All results are expressed as mean ± S.E.M. with n the number of cells analyzed.

3.7 Statistical analysis

Cell-ELISA data were statistical analyzed using ANOVA test and patch clamp data with Mann-Whitney U test, respectively.

3. Results

3.1 Characterization of high and low Kv2.1expressing *Ltk*$^-$ cell lines

Two stable Kv2.1 expressing *Ltk*$^-$ cell lines were developed; a high (525 ± 70 pA/pF at +20 mV, n = 12) and a low (150 ± 25 pA/pF at +20 mV, *n* = 8) expressing cell line (Fig. 1A-B). Whereas the high Kv2.1 expresser was used for immunization of the dromedary to induce the humoral immune response, against the Kv2.1 channel proteins, the low Kv2.1 expresser was used for electrophysiological recordings to secure voltage control. Characterizing the voltage dependence of Kv2.1 channel activation yielded a conduction versus voltage (GV) curve with a midpoint potential $V_{1/2}$ of -2.0 ± 0.3 (*n* = 8) mV and a slope factor *k* of 8.6 ± 0.3 mV (Fig. 1C). These values, as well as the kinetics of channel activation (fig. 1D) were similar to values reported in literature for the Kv2.1 channel [16, 17]. Another characteristic hallmark of Kv2.1 channels is its U-type inactivation process [18, 19]. The voltage dependence of channel inactivation (Fig. 1E) displayed this U-shaped inactivation profile and yielded an inactivation curve with a $V_{1/2}$ of -24.5 ± 2.2 (*n* = 8) mV and a slope factor *k* of 5.2 ± 0.1 mV (Fig. 1F). Since the biophysical properties of the Kv2.1 currents in our cell lines resemble the Kv2.1 data in literature [16-19], we concluded that our cell lines stably express the Kv2.1 channel correctly.

3.2 Standardized of seeded *Ltk*$^-$ cells overexpressing Kv2.1 channel

Five wells of a 96 maxisorb plate were seeded with *Ltk*$^-$cells overexpressing Kv2.1 channel and blank *Ltk*$^-$ cells at roughly 10,000 cells per well and cultured overnight. The assay was performed as described in the Materials and Methods section. The mean and the standard deviation of the measured optical densities for the five wells correspond to 0.229 ± 0.018 for the blank *Ltk*$^-$cells and 0.243 ± 0.023 for *Ltk*$^-$cells overexpressing Kv2.1 channel, respectively. These results show that before Cell-ELISA testing, we had approximately the same number of cells.

3.3 Monitoring of anti Kv2.1 immune response in dromedary

A dromedary was immunized with *Ltk*$^-$cells overexpressing Kv2.1 channels. Blood was collected at days 0, 7, 21 and 51 to prepare serum that was stored at -80°C until further use. Subsequently, the induction of an immune response was evaluated by testing the sera before and after immunization by (whole cell-) ELISA. The results in figure 2 clearly show an increasing titer of cell-specific antibodies where the serum of Day 51 yielded the highest absorbance (OD = 1.55) at a 1:5000 dilution as revealed by the rabbit anti-dromedary Ig antiserum (1:8000 in PBS) and goat anti-rabbit Peroxidase conjugate (1:1000 dilution). Apparently, this high immune response was obtained after only one primary injection in complete Freunds adjuvant and three boosts in incomplete Freunds adjuvant, each time mixed with 10^8 stably Kv2.1expressing cells. Monitoring the Kv2.1 channel-specific serum titer by subtracting the signal for blank *Ltk*$^-$cells from that of *Ltk*$^-$cells expressing Kv2.1 channels, we observed that this Kv2.1-specific immune response was significant in serum of Day 51. After subtracting the measured absorbance of blank *Ltk* that didn't express the Kv2.1 channel (control), the remaining specific OD corresponds to 0.4 OD units/ml (corresponding to a signal increase by approximately 30%). The results obtained with the Cell-ELISA showed a statistically significant difference in cell recognition between the two curves ($P < 0.05$). These data confirm (i) the high immunogenicity of cells in general and (ii) the particular immune reactivity directed against the Kv2.1 channels exposed on the surface of *Ltk*$^-$ cells expressing the channel (Fig. 2).

3.4 Effect of Day 51 serum on Kv2.1 specific currents

Patch clamp experiments were performed on the stably transfected *Ltk*$^-$cell line that moderately expressed the Kv2.1 channels (the low Kv2.1 expresser). To explore the effect of the immune serum on Kv2.1 currents, the cells were incubated for 2 hours with the serum (diluted 1:50 in cell medium) collected at Day 0 (i.e. pre-immune serum), which was obtained

before the immunization process and consequently did not contain Kv2.1 specific antibodies, and at Day 51 before performing the patch clamp experiments. After the incubation period the steady-state current amplitude at +40 mV was recorded and normalized to the cell capacitance (Fig. 3). This current density analysis indicated that cells incubated with serum of Day 51 displayed a statistically significant reduction in current amplitude compared to cells incubated with the serum of Day 0 (approximately a 2-fold reduction) and even compared to untreated cells (Fig. 3B), i.e. cells without pre-incubation of serum that reflect the current density of the stable cell line. Although serum contains, besides antibodies, plenty of other components that may affect Kv2.1 channels, the current density of cells incubated with serum of Day 0 was remarkably similar or even slightly increased (not statistically significant) compared to untreated cells, which were kept in standard culture medium (Fig. 3B).

Approximating the activating current at +40 mV depolarization with a single exponential function yielded a time constant of 30.0 ± 1.8 ms (n = 31) after pre-incubation with serum of Day 51. This value was similar to the time constant obtained in untreated cells (control conditions), being 31.4 ± 1.4 ms (n = 49). Analyzing the speed of current deactivation at -25 mV yielded a time constant of 122 ± 4 ms and 114 ± 7 ms for control conditions and upon incubation of cells with serum of Day 51, respectively. This analysis indicated that the serum collected at Day 51 did not induce important changes in the kinetics of channel opening or closure (Fig. 3.A). Thus, cells incubated with serum of Day 51 displayed a statistically significant reduction in current density compared to cells incubated with serum of Day 0 and untreated cells kept in control conditions. However, the remaining currents displayed kinetics similar to those of both untreated cells (control conditions) and cells incubated with serum of Day 0, which displayed time constants of 24.9 ± 1.5ms and 100 ± 9ms (n = 8) at +40mV and -25 mV, respectively. Collectively, these data indicate that the serum of Day 51 contains

antibodies that specifically reduced the Kv2.1 current amplitude without substantially affecting the channel kinetics.

4. Discussion

Malfunctioning and/or overexpression of Kv2.1 channels have been observed in several tumor cells, particularly in gastric cancer cells (i.e. SGC7901) including gastric epithelial cells [4, 5]. In the present study, our main objective was to develop Kv2.1-specific antibodies recognizing the extracellular regions of the channel as present within the intact protein on cells, rather than binding to (synthetic) oligopeptide fragments of the Kv2.1 channel that fail to reflect the native conformation of the target. To this end, one dromedary was immunized with cells that stably express functional Kv2.1 channel proteins at high density (Fig. 1). This strategy yielded a specific immune serum with antibodies that were most likely directed against the extracellular regions of Kv2.1. As expected, the immune sera, tested by Cell-ELISA, indicated a steadily increasing titer of antibodies recognizing epitopes on the *Ltk*$^-$ cells. Moreover, it is clear that a measurable fraction of antibodies in the serum of Day 51 associate specifically to the Kv2.1 channel proteins as the signal on *Ltk*$^-$ cells expressing Kv2.1 is 30% higher compared to that of Blank *Ltk*$^-$ cells (at a serum dilution of 1:5000). The pre-immune serum taken at Day 0 (at a 1:5000 dilution) did not contain Kv2.1 channel-specific antibodies at a detectable level (Fig. 2).

Recording Kv2.1 currents in whole cell voltage-clamp modus of the patch-clamp technique demonstrated the ability of components within Day 51 serum to decrease the potassium flux. Interestingly, 2 hours pre-incubation with a 2% v/v solution of serum in culture medium resulted in a statistical significant 2-fold reduction in current density (Fig. 3). Since no changes in channel gating kinetics were observed, the antibodies of Day 51 serum most likely obstruct ion permeation by physically occluding the extracellular pore mouth although an allosteric modulation cannot be completely excluded.

Recently, Jin Li et al. [20] reported Anti-KCNQ1 autoantibodies found in a subgroup of patients with dilated cardiomyopathy (which is associated with QT interval shortening) increased the I_{Ks} current. They investigated the functional modulation of I_{Ks} current density in HEK 293 cells upon exposure to serum containing anti-KCNQ1 autoantibodies. In contrast with our results, they reported a significant enhancement in current (2.7 fold increase in mean I_{Ks} current density, respectively). However, similarly to our approach the serum with anti-KCNQ1 antibodies was exposed to the cells for 1 hour before performing the patch clamp experiment. Therefore, the antibodies do most likely recognize extracellular parts of the KCNQ1 channel, which strengthens that antibodies operating via extracellular regions are capable of modulating the channel behavior.

Previously, a rabbit anti-Kv2.1 antibody was developed that selectively and potently inhibited Kv2.1 channels [21]. Transiently expressed Kv2.1 currents in COS-1 cells were inhibited by approximately 50%, after a 10 min exposure to 3 nM rabbit anti-Kv2.1 antibody within the patch pipette [10]. However, in contrast to our data this inhibiting antibody operates via the intracellular side and needs to be applied through the patch-pipette. To the best of our knowledge, our report is the first description of an anti-Kv2.1 antibody that modulates the Kv2.1 current by interacting with extracellular epitopes of the channel.

The therapeutic potential of antibody based channel modulators is evidenced by the recent report of the E314 polyclonal antibody that specifically recognizes and blocks the human Kv1.3 channel. Most importantly, the E314 antibody is considered safe to be used, which makes it a clinically promising human Kv1.3 channel blocker to treat inflammatory diseases [13]. Major advances in antibody technologies have been made over the last decades and there is a substantial growth in the list of active antibodies targeting voltage-gated ion channels [12]. To our knowledge, this is the first report describing the successful generation of an anti-Kv2.1 immune serum in dromedary containing antibodies that selectively target expressed

Kv2.1 channels with some exerting an inhibiting effect on the current density by binding to extracellular epitopes of the channel. Therefore, our study is useful to develop a dromedary-serum-based Kv2.1 detection kit and specific binders (i.e. non-modulators, modulators and/or blockers) for further rational design of channelopathy treatment.

5. Conclusions

We report a successful strategy of induce in dromedary a specific immune response against a potassium channel subtype expressed in an active conformation on the surface of heterologous cells. This work forms a good basis to seek subsequently for recombinant antigen-binding fragments, which possibly modulate potassium channels and in particular the Kv2.1 channel subtype.

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Supporting Information

Legends to figures

Fig.1. Biophysical properties of the high and low Kv2.1 expressing cell line

A, Representative ionic current recordings of blank *Ltk*$^-$ cells (left) and two different *Ltk*$^-$ cell lines that were stably transfected with Kv2.1 cDNA. Both cell lines express the channel differently yielding a low current phenotype (middle to low Kv2.1expresser) and a high current phenotype (right, high Kv2.1 expresser). Currents were elicited using the pulse protocol shown on top and zero current level is indicated by the horizontal bar at the start of the current recordings. **B**, Bar chart showing the average current density ± SEM for the low (left) and high (right) Kv2.1 expressing *Ltk*$^-$ cell line. The current density was calculated by normalizing the steady-state current amplitude at + 40 mV, obtained from recordings as shown in panel A, to the cell capacity, which is a measure for cell size. **C**, Conduction versus voltage GV curve of the Kv2.1 current in the low Kv2.1 expresser, which was obtained by plotting the normalized tail current amplitude from pulse protocols shown in panel A as a function of pre-pulse potential (data points are average ± SEM). Solid line represents the average fit with a Boltzmann equation. **D**, Plot shows the average voltage-dependent activation time constants ± SEM for the low Kv2.1 expresser (circles, n = 8). **E**, On top, the typical voltage protocol that was used to determine the voltage dependence of channel inactivation. After applying a 5 s depolarizing step the amount of channel inactivation was quantified by applying a +60 mV test potential. Below a representative ionic current recording of the low Kv2.1 expressing cell line. Note the gradual reduction in current upon prolonged depolarizing potentials, which reflects channel inactivation. **F**, Panel shows the voltage dependence of channel inactivation, which was obtained by plotting the normalized current during the +60 mV test potential from pulse protocols shown in panel E as a function of

depolarizing pre-pulse. Note the Kv2.1 characteristic U-type inactivation profile with reduced inactivation at more depolarized potentials.

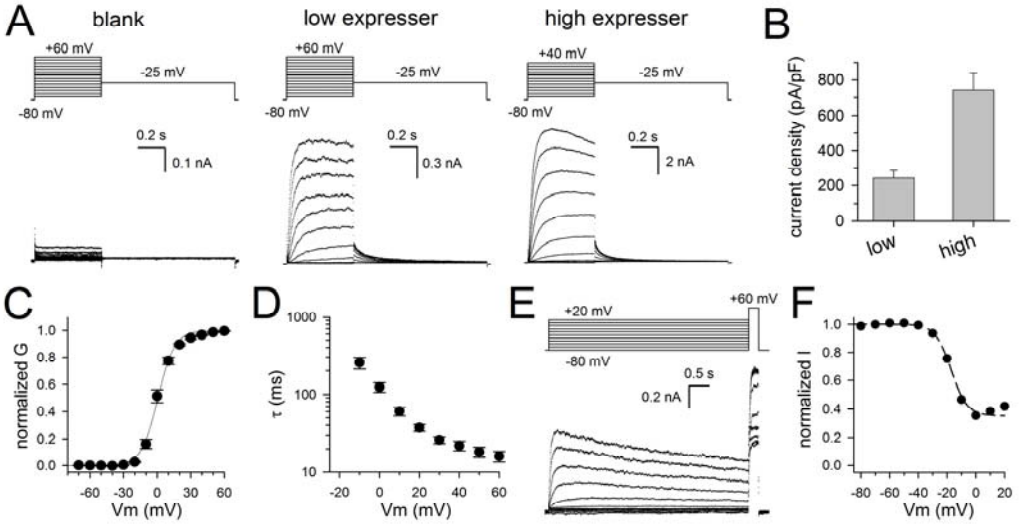

Fig.2. Dromedary immune response to Kv2.1channels during the immunization

Immune dromedary sera of Day 0, 7, 21 and 51 diluted 1:5000 were tested by Cell-ELISA using the *Ltk*$^-$ cells overexpressing Kv2.1 as target. Naïve *Ltk*$^-$cells (blank) were used as control. Presence of bound antibody was revealed using an anti-dromedary IgG rabbit serum (1:8000) and a goat anti-rabbit IgG peroxidase conjugate (1:5000). After adding substrate, optical density was measured at 492 nm.

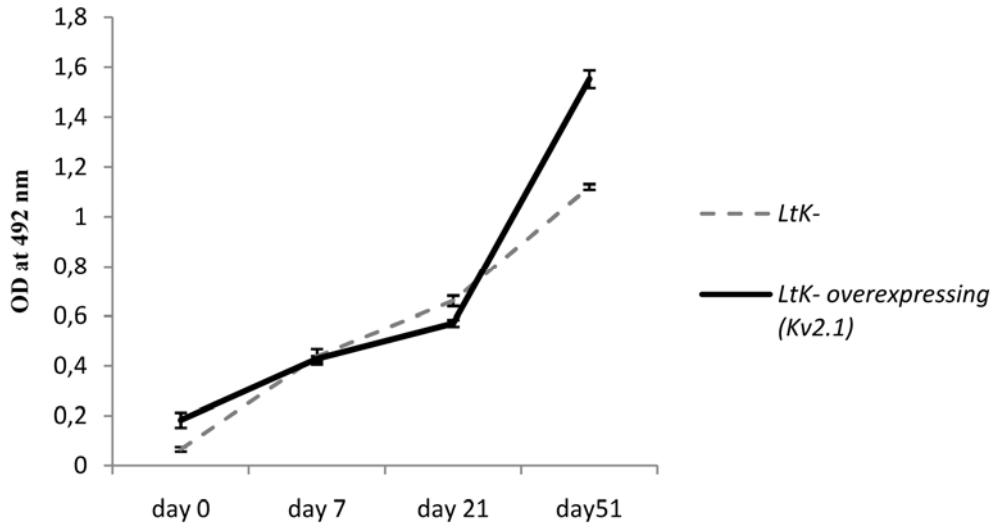

Fig.3. Patch clamp recording of the currents of Kv2.1 channels upon pre-incubation with serum of Day51

A, Representative Kv2.1 currents recorded from low Kv2.1 expressing *Ltk*$^-$ cells that were either untreated grown under control(gray recording) or upon a 2 hours incubation with serum of Day 51 (black trace). Currents were elicited using the pulse protocol shown on top. Note that the kinetics of the black and gray trace were similar indicating that the serum did not induce obvious changes in the speed of current activation or deactivation. **B**, Bar chart shows on the left the average current density ± SEM for the low Kv2.1 expressing *Ltk*$^-$ cell line grown under control conditions (untreated cells, n = 49) and after 2 hours incubation with serum from Day 51 (middle, n = 31) or serum from Day 0 (right, n = 8), the latter was used as a blank. The current density was calculated by normalizing the steady-state current amplitude at + 40 mV to the cell capacity. Note that cells incubated with serum from Day 51 displayed a statistically significant (* $p < 0.05$ and ** $p < 0.03$) reduction in current density compared to both untreated cells kept in control condition and cells incubated with serum from Day 0 (blank).