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$K_V1.3$ and $K_V1.5$ channels in leukocytes

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This PhD Thesis has been performed under the direction of Dr. **Antonio Felipe Campo** in the *Molecular Phisiology* laboratory in the Department of Biochemistry and Molecular Biomedicine, Institute of Biomedicince (IBUB), Faculty of Biology, University of Barcelona.

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PhD Thesis
University of Barcelona, Faculty of Biology
Department of Biochemistry and Molecular Biomedicine
Doctoral programme in Biomedicine
2016, Barcelona

ACKNOWLEDGMENTS

ACKNOWLEDGMENTS

L'aigua de la dutxa em desperta de cop, i el mosaic de les rajoles en forma de membrana plasmàtica amb els seus canals i els seus ions em recorda que he d'espavilar. Avui no em dóna temps d'esmorzar amb tu, Toni, però ja tindrem temps, molt de temps. De camí a la universitat, en metro, dubto entre agafar el ple de significat llibre de l'Amin Maalouf que van regalar-me els incondicionals dels bioquímics (la Leti, el Loren, l'Isaac, la Laura, el Facun, el Marcel, la Daisy, l'Elena, et al. i el Jordi) o mirar-me els deures d'àrab per a que, almenys per un cop, amb el Marc i l'Elena no haguem d'improvisar quelcom amb riures de fons. A la sortida del metro em trobo amb l'Antonio *petit*, que és la perseverança personificada, tot i les habituals avaries de la Renfe que m'explica. A l'entrada del departament ens trobem al Toni qui, dia sí dia també, i no falla, ens delecta amb un acudit. Ràpidament, i abans de marxar cap a citometria per a que en Jaume o qualsevol dels habituals m'ajudin a analitzar les dades del *sorting*, baixo a la sala de cultius a electroporar uns limfòcits per a demà mentre el Xico, el Viel, l'Olga, el Cesc, la Laura, l'Ibon, la Ingrid, el Ricardo, la Liska, l'Ester, la Lorena, la Cristina o la resta de TAM, RST i INS fan que, amb les seves converses i bromes, cultius sigui un lloc fresc, jove, amable i ple de riures i màgia.

A mig matí surto del despatx de l'Antonio, content i agraït pel fet de sortir molt més motivat de com havia entrat, i, de camí al laboratori, noto la presència de la Kasia que em segueix, com sempre, a uns metres de distància. Però també com sempre, allà hi és. Al laboratori, just acaba de sonar el *timer*, i trec unes mostres de cDNA d'un experiment d'activació de macròfags que havien estat incubant-se amb la retrotranscriptasa mentre noto com la Laura m'observa per sobre de les seves ulleres i amb un somriure des del seu taulell. Després, analitzo unes dades de proliferació mentre fem temps per a anar a dinar. Pensant en proliferació, no puc evitar posar-me nostàlgic i rememorar la bella i històrica localitat de Göttingen, on gràcies a la tecnologia i el caliu de tota aquella colla de científics, des del Luis i la Joasia, fins a la Diana, l'Adam, la Farrah o la resta de la tropa, vaig viure una primavera ben especial. Aprofito també a mirar el correu electrònic i observo la sempre inspiradora crònica de l'Àlvaro des de vés-a-saber-on però alhora tan a prop, i també responc algun correu electrònic a la Raquel o Vanessa, o la resta de secretàries amb alguna bonica frase que ens faci riure recíprocament. Miro el telèfon, responc amb un *sms* al sempre present i pacífic Jordi, i després me n'adono de quan tard és. La Sara i jo tenim molta gana i estem desesperant-nos. Ens mirem i ens ho diem absolutament tot. Fem sinèrgicament les subtils bromes pertinents i aconseguim, plegats, que de sobte, tot el laboratori, rient i embriagat de bon rotllo, vagi a dinar. Agafo el *tupper* amb les croquetes de la iaia, el sabor de les quals serà perenne i sempre present, com ella.

Quan tornem al laboratori, sona la cançó de *Líquido Narcótico* que han deixat posada el Ruben, la Txell, la Núria, la Joasia, el Ramón i altres. També, per sort, han deixat moltes altres coses a MP. Mentre la Sara canvia l'estil de música i jo carrego proteïnes de diferents leucòcits per a detectar KCNE4 en els gels de western que algun tècnic –no recordo si la Patricia, l'Edgar o qui– ha preparat aquest matí, la Caveia m'informa, amb un somriure més gran que els decibels del mateix, que, tot i que no sigui estiu, la Concepció vindrà demà amb ratolins i a donar-nos un cop de mà. Per a acabar d'adobar el que cada cop s'assembla més a qualsevol tarda de divendres, entra la que faltava per al duro, l'Anna,

amb un cogombre a la mà i un altre somriure als llavis, ja que acaba de recollir les verdures. A les fosques perquè algú està fent unes immunocitoquímiques, o unes *plasma membrane loans*, o un pas crític d'un protocol d'ELISA, o per qüestions ètico-mediambientals, o perquè el fluorescent no funciona, ara no recordo ben bé el motiu, treballem en un atmosfera immillorable i sense parar de riure. De sobte, l'Antonio entra al laboratori i, tot mirant de reüll els films de western blot que hi ha sobre el transil·luminador, arregla el fluorescent pujat a una cadira que sembla la Laia fent una de les seves postures de ioga, alhora que comenta la darrera pel·lícula que ha vist, aquella on surt la Carla complint el seu somni, americà o no.

Després de valorar el contingut d'IL-2 d'una placa d'ELISA a la sala d'aparells -el resultat ha estat positiu, però no espectacular com aquell dia de Sant Jordi!-, m'acomiado de la Núria, que està a la sala de *patch*, lluitant com sempre per obtenir un registre amb una de les tècniques més boniques i sacrificades que he conegut. Abans de marxar, els txaperons m'ajuden a posar unes midis a créixer i no puc evitar riure quan recordo la combinació

d'ukeleles i midis amb la Marta i el Martí. Ric tant que la Jesusa i la Clara es foten de mi. Bé, no, elles mai ho farien i, en canvi, ho trobarien tendre i *summerlòvic*. De ben segur que es tracta dels avispats del Dani i la Irene. Quins parells! Quan surto de la sala del *shaker*, saludo des de la distància a l'Ibrahim de BQI o al David de Neuro, no el distingeixo bé. Qui segur que no seria era el Pere d'LPL, que avui havia marxat corrent, avui sense les meves claus.

Torno a agafar el metro per a dirigir-me a algun indret de la guapa i poderosa ciutat de Barcelona. Per sort, cada dia i cada època em duen i em descobreixen, de forma espontània o racional, un nou lloc, divers i diferent. Per això dubto. I encara dubto si baixaré a Sant Andreu amb la Cèlia, si m'acolliran els Hipolitians amb el seu humanisme intrínsec, si avui esdevindrà un capvespre mediterrani i màgic amb la Sara, si he de baixar a la llar familiar que hem construït amb la Bea i sobretot amb la Núria i l'Aran, o bé si acabaré anant fins a Nou Barris on, els autèntics i els de sempre, la mama, el papa i la Marta, segur que m'espren.

Finalment, i com en el fons em tornen a inspirar les proteïnes del mosaic, que els dies i la vida segueixin fluint i dansant al ritme d'aquest dinàmic equilibri homeostàtic.

Gràcies.

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ALCL	Anaplastic large cell lymphoma
APC	Antigen presenting cell
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BM	Bone marrow
Cdk	Cyclin-dependent kinases
cDNA	Complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
CNS	Central nervous system
CRAC	Ca^{2+} release activated channel
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
F	Follicular lymphoma
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FRET	Försters resonance energy transfer
FSC	Forward scatter
HGNC	HUGO Gene Nomenclature Committee
HRP	Horseradish peroxidase
Hscore	Histoscore
IC_{50}	Half maximal inhibitory concentration
Ig	Immunoglobuline
IL	Interleukin
IS	Immunological synapse
IUPHAR	International Union of Pharmacology
KCNA	Potassium voltage-gated channel subfamily A
KCNE	Potassium voltage-gated channel subfamily E
kDa	kilodalton
K_v channel	Voltage-dependent potassium channel
LN	Lymph node
LPS	Lipopolysaccharide
MCL	Mantle cell lymphoma
MgTX	Margatoxin
MHC	Major Histocompatibility Complex
mRNA	Messenger ribonucleic acid

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PI	Propidium iodide
PMT	Photomultiplier
Psora-4	5-(4-Phenylbutoxy)psoralen
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Sideward scatter
T	T cell lymphoma
TBS	Tris-buffered saline
TCR	T cell receptor
TEA	Tetraethylammonium
T_{CM}	Central memory T lymphocyte
T_{EM}	Effector memory T lymphocyte
TM	Transmembrane
TNF-α	Tumor necrosis factor α
WT	Wild type
YFP	Yellow fluorescent protein
4-AP	4-aminopyridine

1. INTRODUCTION

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1.1. Ion channels

1.1.1. Biophysical generalities

Homeostasis is the tendency to keep the equilibrium and the internal stability in the different biological systems. Claude Bernard, the father of the experimental medicine who introduced the concept in 1865, said that "homeostasis is the dynamic equilibrium that keeps us alive". Life greatly depends on excitability, which is defined as the capacity to react to changes of the environment with changes in the equilibrium of matter and energy, according to the nervous system researcher Verwom in 1899. Excitability is linked to electric potential or membrane potential, because when a stimulus is provoked experimentally by exciting cells, electric potential changes. The bigger the stimulus strength, the bigger the change in membrane potential (Hille 2001; Bezanilla 2008; Armstrong and Hille 1998).

Most cells have a shell of negative charges on the cytoplasmic side of the membrane and a shell of positive charges in the extracellular side. This charge separation creates a potential difference or voltage across the membrane, and it is called membrane potential. Electrochemical potentials are produced when a semipermeable membrane separates asymmetric ion concentrations. The origin of this membrane potential is the presence of membrane non-permeant and negatively charged organic molecules in the cytosol. The presence of additional anions in the intracellular compartment favours a greater concentration of cations. The movement of ions continues until equilibrium is reached, where the tendency of ions to move down the concentration gradient into or out if the cell is compensated by the electrical gradient, known as the Gibbs-Donnan equilibrium (Kew and Davies 2009).

All systems try to reach equilibrium, a state in which the tendency for further changes disappears although forward and backward fluxes in every microscopic transport mechanism or chemical reaction are equal but persist. If Boltzmann equation of statistical mechanics which describes the equilibrium distribution of particles in a force field is adapted to chemistry, then the resulting equation is the one theorized and demonstrated in 1888 in Göttingen by the physician Walther Hermann Nernst. According to the Nernst equation (Fig. 1), ionic equilibrium potentials vary linearly with the absolute temperature and logarithmically with the ion concentration ratio. The potential level across the cell membrane that exactly opposes net diffusion of a particular ion through the membrane is called the Nernst potential or equilibrium potential ($E_{eq.x}$) for that ion (Hille 2001).

$$E_{eq.,x} = \frac{RT}{zF} \ln\left(\frac{[X]_{out}}{[X]_{in}}\right)$$

Figure 1. Nernst equation. Where $E_{eq.x}$ is the equilibrium potential for the ion, R is the gas constant, T is the absolute temperature (273.13 + ºC), z is the valence of the ion, F is the Faraday's constant, $[X]_{out}$ is concentration of ion outside the cell and $[X]_{in}$ is concentration of ion inside the cell.

Both electroneutrality and osmolarity (total ionic concentration) in the intra and extracellular medium are the same, although ionic compositions are different in both sides of the membrane. In general, sodium and chloride ion concentrations are 10-times higher in the extracellular medium than in the intracellular one. Oppositely, potassium ions are more abundant intracellular. Unlike the others, calcium ions are presented at really low concentrations in the cytosol, but they do have a crucial role in cell

signalling and functioning as a universal second messenger. The resting membrane potential of a cell is determined by the presence of ion channels in the membrane and by the asymmetric distribution of ions inside and outside of the cell. It is important to point out also the presence of ion pumps that build up ionic concentration gradients. The active transporter Na^+/K^+ ATPase has a major role in maintaining the electrochemical gradient and a high potassium concentration inside the cell.

Opened pores of ion channels allow some types of ions to flow passively down their electrochemical activity gradients at a very high rate (>10^6 ions per second). Ion channels have the important property of selective permeability, allowing some restricted class of small ions to flow through their pores, but ion channels are not neither perfectly selective nor constantly opened. Permeability (p) of an ion determines its contribution to the membrane potential. At physiological conditions, the resting membrane potential can be determined from the commonly named Goldman equation (Fig. 2), which describes the steady-state inter-diffusion of ions away from equilibrium. Besides considering total ionic concentrations, this equation contemplates also the relative permeability of each ion. For that reason, those ions with a higher conductance or more open channels (K^+ channels in most cells) would dominate and determine the resting membrane potential. Because K^+ is more permeant than Na^+ or Cl^-, and due to the fact that its Nernst equilibrium potential reaches negative values, the cell membrane potential will be, generally, negative, around -90 mV. Net electric currents or charges movement across the membrane have an immediate effect on membrane potential. In turn, other voltage-gated channels in the membrane detect the change in membrane potential and become excited. This is how the electric response is made regenerative and self-propagating. Electricity is the means to propagate the signal to a point where a nonelectrical response is generated such as changing the internal Ca^{2+} ion concentration (Hille 2001; Kandel et al. 2013).

$$V_m = \frac{RT}{F} \ln\left(\frac{p_K[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_K[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o} \right)$$

Figure 2. Goldman-Hodgkin-Katz equation. Where R is the gas constant, T is the absolute temperature (273.13 + °C), F is the Faraday's constant, p_k, p_{Na} and p_{Cl} are the membrane relative permeabilities for K^+, Na^+ and Cl^-, respectively, with p_k having the reference value of one.

The size of the current is determined by the potential difference between the electrodes and the electrical conductance of the solution between them. Conductance is the measure of the ease of flow of current between two points, and it is the reciprocal of the resistance, measured in ohms. Ohm's law is the relation between current (I), voltage (E) and conductance (g) or resistance (R): $E = I \times R$ or $I = g \times E$. Ohm's law plays a central role in membrane biophysics because each ion channel is an elementary conductor embedded in an insulating lipid membrane that presents an energy barrier to ion crossing. The total electrical conductance of a membrane is the sum (macroscopic currents) of all these elementary conductances (single-channel currents) in parallel.

The lipid bilayer of biological membranes separates internal and external conducting solutions by an extremely thin insulating layer, which physicians describe as an electrical capacitor. Capacitance (Fig. 3), measured in farads, is a measure of how much charge needs to be transferred from one conductor to another to set up a given potential difference between them. The capacity to store charges arises from their mutual attraction across the gap and by the polarization they develop in the intervening insulating medium. Capacitance depends on both the dielectric constant of that medium and on the geometry of the conductors on either side. Cell membranes have a specific capacitance near 1.0 $\mu F/cm^2$. Assuming that the dielectric constant of

hydrocarbon chains is 2.1 and that the thickness d of the insulating part of the bilayer is only 23 Å, consequently the molecular dimensions and surface of the biological membranes can be calculated from the electrical capacitance.

$$C = \frac{Q}{E} = \frac{\varepsilon \times A}{d}$$

Figure 3. Capacitance equations.

Electrophysiology currents from single ion channels are recorded by means of the patch-clamp technique, developed in 1976 by Erwin Neher and Bert Sakmann. Albeit the flux of ions through ion channels is passive, in some open channels the current varies linearly with the driving force (ohmic channels), while in others, the current is a nonlinear function of the driving force (rectifying channels). The latter type conducts ions more readily in one direction than in the other because of the asymmetry in the channel's structure or environment. When there is a net flow of cations or anions into or out of the cell, the charge separation across the resting membrane is disturbed, altering the electrical potential of the membrane. A reduction of charge separation, leading to a less negative membrane potential, is called depolarization, whereas an increase in charge separation is called hyperpolarisation (Kandel et al. 2013).

1.1.2. Classification

Ion channels are transmembrane proteins that conduct the movement of ions from one side of a membrane to the other. They are single, repetitive-structure proteins or multiple subunits arranged to essentially form a pore through the plane of the membrane. These proteins are named as α subunits if they are the primary pore forming subunits and β or γ subunits if they are auxiliary ones. Ion channels can be classified according to the ion for which they are mainly permeable (Na^+, K^+, Cl^-, Ca^{2+} channels), according to their activation mechanism (voltage-gated, ligand-gated or other categories such as mechanosensitive, temperature, pH or second messenger activation) (Kew and Davies 2009).

Potassium-selective channels are the largest and most diverse group of ion channels, represented by more than 70 genes in mammalians (Gutman et al. 2005). The vast majority of potassium channels is subdivided into three subfamilies (Fig. 4) according to their structure: the two transmembrane (2TM), 4TM and 6TM channels (Yu et al. 2005). A) Members of the 2TM family are also known as inwardly-rectifying potassium (K_{ir}) channels. Inward rectifiers conduct more current when the transmembrane potential is hyperpolarized than when it is depolarized from the potassium equilibrium potential by an equivalent amount. Inward rectifiers mediate transport across the cell membrane and stabilize the resting potential near the potassium equilibrium potential. This family consists of seven members, including the strong inward-rectifier channels, the G-protein-activated inward-rectifier channels and the ATP-sensitive channels. B) The 4TM family is believed to underlie many leak currents in native cells. These channels tend to be open at a range of voltage potentials and are widely regulated by neurotransmitters and other biochemical regulators. Subtypes include TWIK, TREK, TASK, TALK and THIK. The basic α subunit is composed by two pore domains, hence forming usually dimers, rather than tetramers, given the often used names for this channel family- "two-pore domain potassium (K2P) channels" or "tandem pore potassium channels". The functions and pathophysiology of these channels are not clear and further research is needed. C) The 6TM family includes the voltage-gated potassium (K_v) channels, the KCNQ channels, the EAG channels (also including the Herg channels) and the calcium-activated potassium (K_{Ca}) channels BK (slo) and SK. The pore forming α subunits of the 6TM subfamily form tetramers. These channels

consist of a single pore loop and intracellular N- and C- termini (Miller 2000; Yu et al. 2005; Coetzee et al. 1999).

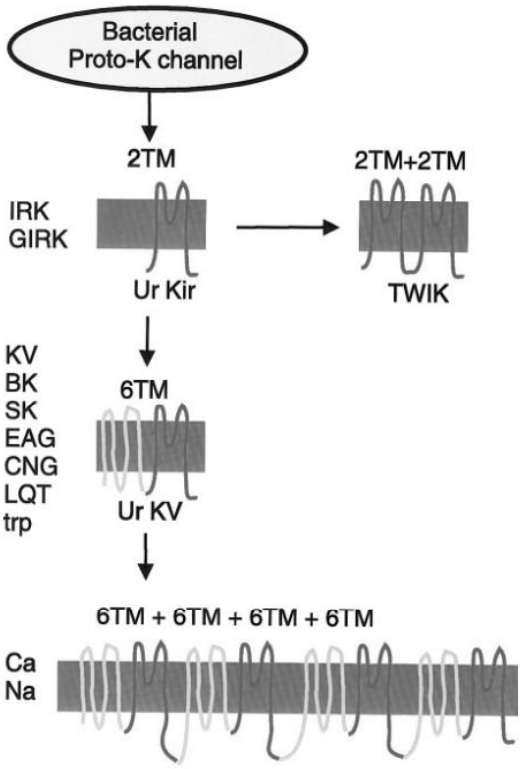

Figure 4. Hypothetical sequence in the evolution of voltage gated ion channels based on their domains structure. Adapted from Armstrong and Hille 1998.

Members of the voltage gated ion channel superfamily are found in all three domains of life: Eukarya, Eubacteria and Archaea; suggesting that these channels are extremely ancient. Recently the subunit of a calcium channel gene that encodes just one subunit of the four required for channel formation was found in the genome of the eubacterium *Bacillus halodurans*. It is suggested to be the missing link between K_v channels and voltage-dependent calcium (Ca_v) channels in higher organisms (Durell and Guy 2001).

1.1.3. Voltage dependent potassium channels

Voltage dependent potassium channels were classified based on amino acid sequence alignments of the entire hydrophobic core of the proteins. Fig. 5 shows the assigned names and the phylogenetic relationship between two different voltage-dependent potassium channel family members: the K_v1-9 and the K_v10-12. The IUPHAR and HGNC names are shown together with the genes' chromosomal localization and other commonly used names.

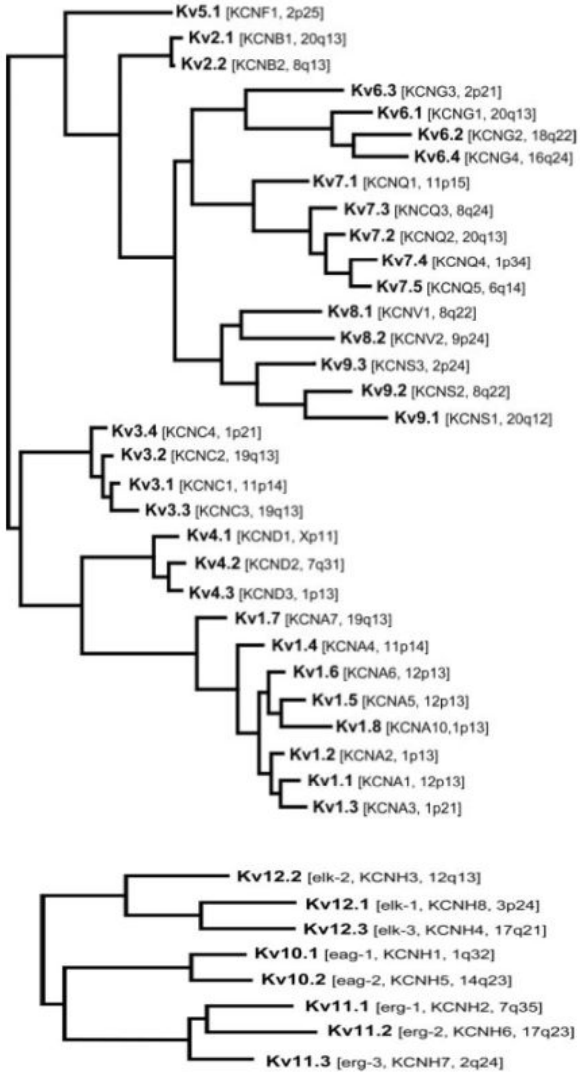

Figure 5. Phylogenetic trees for K_v1-9 and K_v10-12 families. The IUPHAR and HGNC names are shown together with the genes' chromosomal localization and other commonly used names. From (Gutman et al. 2005).

It was in 1987 when the first genes for K_v channels were isolated as naturally occurring mutations of the Shaker locus in the fruit fly *Drosophila melanogaster*. As a result, K_v channels are usually referred to as Shaker-like channels. Three related

genes, Shal, Shaw and Shab were also cloned from the same animal model. The mammalian homologs of all four genes are K_v1 (Shaker), K_v2 (Shab), K_v3 (Shaw) and K_v4 (Shal). Epilepsy, episodic ataxia/myokimia and episodic cardiac arrhythmia (the long QT syndrome) are caused by mutations in genes encoding voltage-dependent potassium channels. They are outward rectifiers because those channels allow current to flow out of the cell, but not in.

Other families of the K_v superfamily are the KCNQ channels (KCNQ1-5), also known as K_v7. They are considered delayed rectifiers, since they do not undergo a fast inactivation. Also, it is important to point out the existence of the electrically silent families of potassium channels K_v5, K_v6, K_v8 and K_v9 because, although they are not well defined, are not able to form functional homomeric channels and they instead form heteromers with K_v2 members. KCNH channels, phylogenetically more distant, are eag1-2 (K_v10), erg1-3 (K_v11) and elk1-3 (K_v12). They exhibit two unique characteristics: a signature gene sequence in the P loop region that differs from other channels, and the presence of a cyclic nucleotide binding domain within the cytoplasmic part of the carboxyl terminal. The calcium-activated potassium channels (K_{Ca}) are characterized because are easily opened by means of the elevation of intracellular calcium, leading to membrane hyperpolarization. Structurally they are divided in two grups: (i) the small or intermediate conductance K^+ channel (SK/IK, KCa1) and KCa2), which are highly sensitive to intracellular calcium, are low conductant, weak or negligible voltage-dependent and interact with calmodulin, and (ii) the high conductance potassium channel (BK or Maxi-K, KCa3), which exist as a tetramer of pore-forming α subunits and important and diverse regulatory β subunits.

The huge diversity of K_v channels exceeds the number of distinct genes that encode them due to several factors (Gutman et al. 2005): (i) *Homo- and Heteromultimerization*. Four peptide subunits, each of them encoded by a single gene, are required to assemble a functional K_v channel. K_v channels can be homotetramers but they can also be heterotetramers formed between different subunits within the same family (as in the K_v1, K_v7, and K_v10 families) or within different families (K_v5, K_v6, K_v8, and K_v9 families encode subunits that do not produce functional channels, but increase the functional diversity by forming heterotetramers with K_v2 family subunits). (ii) *Alternative mRNA splicing*. Although some K_v channel genes are known to contain intronless coding regions (K_v1 family with the exception of $K_v1.7$), several members of the K_v3, K_v4, K_v6, K_v7, K_v9, K_v10, and K_v11 gene families contain exons and introns which provide another source of channe diversity. (iii) *Post-translational modifications*. Many K_v channels can be post-translationally modified by phosphorylation, ubiquitinylation, palmitoylation, sumoylation, nitrosylation and oxidation, which in turn increases channel function (Kanda and Abbott 2012). (iv) *Accessory proteins.* A variety of other peptides such as β-subunits, KCNEs, KChIps, KChAPs, DDPLs, AKAP, calmodulin and some others, associate with K_v tetramers and modify their trafficking or biophysical properties.

K_v channels are found in some prokaryotes, such as K_vAP and K_vLm from the archaea *Aeropyrum perni* and the bacteria *Listeria monocytogenes*, respectively. Putative K_v channels are found in choanoflagellates and in basal metazoans, but not in sponges (although it has been reported the presence of other K^+ channels, such as K_{ir}). Cnidarians, considered to be the earliest animals with a nervous system, express multiple K_v channels. By virtue of the fact that seven out of eight mammalian *Shaker* genes that are a result of vertebrate specific gene duplication are intron-less and also because the *Shaker* gene in the sea urchin deuterostome model *Strongylocentrotus purpuratus* is also an intron-less, it is prone to speculate that chordate and most cnidarian

Shaker genes may have evolved from a common intron-less ancestor (Moran et al. 2015).

1.1.3.1. Structure

Rod MacKinnon and his colleagues provided the first high-resolution X-ray crystallographic analysis of the molecular architecture of the bacterial potassium channel KcsA. This technique, that provides a detailed view of the 3D structure of proteins, reported that the channel was made up of four identical subunits arranged symmetrically around a central pore (Doyle et al. 1998). X-ray crystal structures of the K_v1.2 channel and a K_v1.2-K_v2.1 chimera showed that a K_v channel subunit is composed of two domains: the voltage-sensing domain (S1-S4 segments) at the periphery of the channel and the pore domain (S5-P-S6 region) at the central axis of the channel. Actually, each α subunit of a K_v channel consists of six α helix transmembrane (TMs) domains (Fig. 6), named segments S1-S6, with a membrane re-entrant loop between the S5 and S6. The ion-conducting pore and selectivity filter are formed by their S5 and S6 segments and the membrane re-entrant pore loop (P) between them. The voltage-sensing domain is located within the S1-S4 segments. In particular, S4 contains repeated motifs of one positively charged amino acid residue followed by two hydrophobic residues and is the main transmembrane voltage-sensing component.

The transmembrane architecture of all voltage-dependent channels is quite similar. The general topology of the Na_v or Ca_v channels is highly similar to K_v channels and mimics the tetramers of the latter. The structural α subunit with the six TMs is repeated four times but linked in the same amino acid sequence to make the 24 TMs polypeptide. These motifs are referred to as *homologous repeat domains*, denoted as I, II, III and IV and each one is equivalent to a single K_v subunit (Kew and Davies 2009; Yu et al. 2005; Barros, Domínguez, and de la Peña 2012; Kandel et al. 2013).

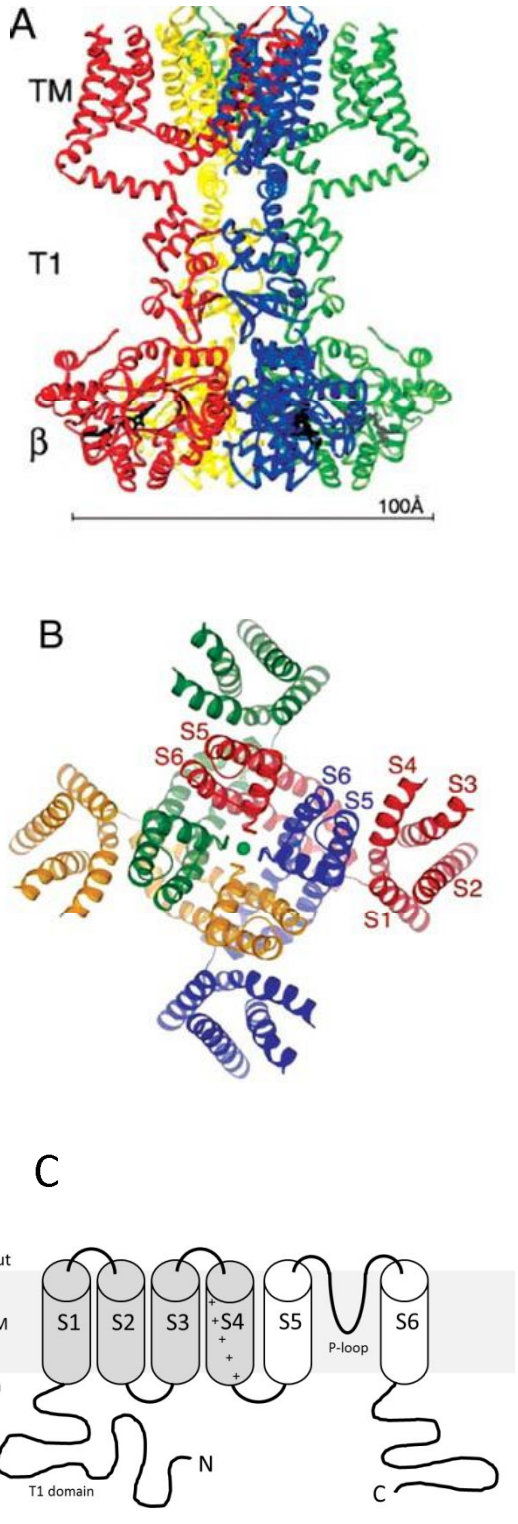

Figure 6. Representation of the tetrameric organization of a K_v channel. Crystal structure of a mammalian voltage-dependent Shaker family K^+ channel (K_v1.2 and the associated β2 auxilliary subunit). Lateral view (A) and top view (B), from Long et al. 2005. (C) Schematic representation of an α-subunit consisting of six transmembrane segments

(S1–S6) and an intracellular N- and C-terminus. The tetramerization domain T1 is situated on the N-terminus, the S1-S4 corresponds to the voltage-sensor domain, and the S5-P-loop-S6 to the pore-domain.

1.1.3.2. Selectivity

It was in 1994 when MacKinnon et al. identified TVGYG as a filter selectivity sequence that confers K^+ channels the property of specifically allow potassium ions to flow through their pores (Heginbotham et al. 1994). The selectivity filter and the cavity residues are highly conserved through various species and channel types; from human to bacterial KcsA-like leakage channel. The presence of negative charges in the selectivity filter pore increases potassium availability at the channel entrance. At the selectivity filter an ion must shed most of its waters of solvation to traverse the channel. Because shedding its waters of solvation is energetically unfavourable, the ion will traverse a channel only is its energy of interaction with the selectivity filter compensates for the loss of the energy of interaction with its waters of hydration (Kandel et al. 2013). Although Na^+ and K^+ monovalent cations possess the same charge and differ only in size, Na^+ has a smaller ionic radius but a larger solvated radius than K^+ (Nightingale Jr. 1959). Therefore, the energetic cost for dehydration is higher for Na^+ ions. Along the walls of the channel, carbonyl groups oxygen atoms create a queue of K^+ binding sites that mimic the water molecules surrounding a hydrated K^+ ion. The fixed filter structure is fine-tuned to coordinate a K^+ better than the smaller dehydratated Na^+. Thus, the selectivity filter is occupied by two K^+ ions alternating between two configurations (Morais-Cabral et al. 2001; MacKinnon 2003; Bezanilla 2008).

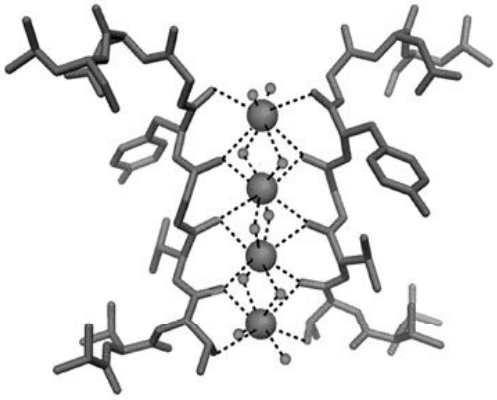

Figure 7. Potassium channel selectivity filter. The polypeptide chain is part of the selectivity filter structure of the ion channel, which uses its main-chain atoms to coordinate the ions. Bigger spheres are potassium ions coordinated by oxygens of carbonyl groups, while smaller balls are water molecules. From MacKinnon et al. 1999.

1.1.3.3. Voltage gating

Channel's response to a stimulus (a membrane potential change, a neurotransmitter or other chemical, a mechanical deformation), called gating, is an apparently opening or closing of the pore. Voltage-dependent or voltage-gated channels are proteins in which some parts of the molecule change their conformation in response to alterations of the electric field within the membrane, shifting the conformational equilibrium back and forth from closed to open (and inactivated) states (Fig. 8) (Barros et al. 2012).

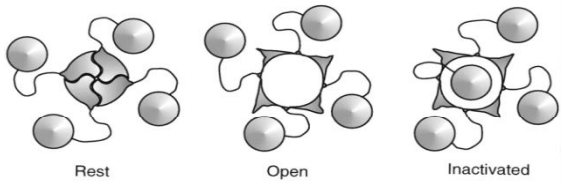

Figure 8. Closed, opened and inactivated conformational states of ion channels. From Armstrong et al. 1998.

Voltage sensitivity is mainly achieved by four positively charged arginine residues on each voltage sensor. Moreover, S4 segment where positive charges are located is otherwise very

hydrophobic. During depolarization of the membrane, the transmembrane segment S4 senses the voltage and moves a limited amount (about 7 Å) across the membrane plane, with a concomitant rotation of as much as 180º and a change of its inclination by about 30º. Movement summation of the four S4 segments gives the impetus for conformational change (Sigworth 2003; Bezanilla 2008; Armstrong and Hille 1998).

1.1.3.4. Inactivation

Inactivation is a process by which an open channel enters a stable non-conducting state after a maintained depolarizing change in membrane potential. Members of the Shaker K^+ channel family can inactivate via two distinct inactivation mechanisms. Whereas (i) the rapid/N-type inactivation behaviour is a consequence of the occlusion of the open pore by an N-terminal tethered cytoplasmic "ball", (ii) the slow inactivation is related to the constriction or collapse of the selectivity filter near the extracellular mouth of the pore. This slow inactivation is composed, first, by a P-type inactivation or closing of the gate at the extracellular end of the pore collapsing the selectivity filter and, secondly, by the C-type inactivation or the conformational change in the region that stabilizes both the non-conducting state and the conformation of the voltage sensors.

In addition to those major types of inactivation, it is important to point out the existence of another type of inactivation in certain types of K^+ channels termed cumulative or "use-dependent". It occurs when inactivation accumulates during repetitive depolarizing pulses delivered at 1 Hz, because recovery during the interpulse interval is incompleted. It is a typical feature of $K_v1.3$ channel, and it is visualized as a reduction in current amplitude with each pulse (Panyi 2005; Barros et al. 2012; Grissmer et al. 1994).

1.1.4. Voltage-dependent potassium channel $K_v1.3$

$K_v1.3$ is an outward delayer rectifying voltage-gated potassium channel. At a DNA level, *kcna3* gene was cloned from brain and it is an intronless coding region located in the chromosome 1p13.3. The $K_v1.3$ promoter contains, besides two SP1 sites and enhancer motifs, a GC-rich fragment and lacks a TATA box, similar to other housekeeping genes or other channels like $K_v1.4$, $K_v1.5$, and $K_v3.1$. The diversity of $K_v1.3$ mRNAs (3.5-9.5 kb) found in lymphocytes and brain may, therefore, result from either alternative splicing in the 3-UTR or alternative use of polyadenylation sites (Simon et al. 1997). From a protein perspective, human $K_v1.3$ pore-forming α subunit is a 523 aminoacid length. As the rest of Shaker channels do, a functional channel is composed of four pore-forming α subunits. Although it can be associated with other Shaker voltage-dependent channels or with some regulatory β subunits, when assembled as a homotetramer it presents several biophysical characteristics.

It was in 1984 when the first electrophysiological evidence about the existence of a voltage-gated potassium channel in human T cells appeared (DeCoursey et al. 1985). It was classified as the normal or n-type potassium channel which, but later in the 1992 it was cloned, identified by homology to the Shaker family of voltage-gated potassium channels, and referred to as the $K_v1.3$ channel. This channel is approximately 1000 times more conductant to K^+ than to Na^+. Its ion selectivity follows: K^+ (1) > Rb^+ (0.77) > $NH4^+$ (0.1) > Cs^+ (0.02) > Na^+ (<0.01). The single channel conductance is 13 pS. The voltage required for activation is -35 mV and it presents a marked C-type inactivation. Its steady-state inactivation parameter h_∞ is -63 mV. The biophysical properties of $K_v1.3$ define an overlap between the activation and inactivation curves that creates a range of membrane voltage where a small and continuous current is present, and it is called

membrane potential window at which the channels can be open at steady-state (Molleman 2002). It is physiologically relevant because this window current overlaps with the resting membrane potential of T cells, which is between −50 mV and −70 mV, thereby underlining the importance of $K_v1.3$ in the membrane potential control of T cells. This channel is blocked by several compounds, but no activator has yet been described (Gutman et al. 2005; Panyi 2005).

Upon depolarization of the membrane $K_v1.3$ channels open rapidly. The short rising phase of the whole cell current is followed by a slow decay during prolonged depolarization as a consequence of inactivation (Fig. 9A). $K_v1.3$ channels lack the N-terminal ball peptide but inactivate by the slow P/C-type inactivation. The inactivation time constant for the whole cell currents at a large depolarization is 200 ms approximately in lymphocytes or in heterologously expressed $K_v1.3$. One feature of $K_v1.3$ that distinguishes it from other channels in the K_v1 family is that it undergoes cumulative inactivation with repeated depolarization (Fig. 9B). Several factors influence the inactivation kinetics of $K_v1.3$ including temperature, phosphorylation by receptor and non-receptor tyrosine kinases, interaction with blockers, the ionic composition of the extracellular solution and the extracellular pH (Panyi 2005).

$K_v1.3$ is expressed and distributed in several tissues, including brain (inferior colliculus > olfactory bulb, pons/medulla > midbrain, superior colliculus, corpus striatum, hippocampus, cerebral cortex), lung, islets, thymus, spleen, lymph node, fibroblasts, B lymphocytes, T lymphocytes, pre-B cells, tonsils, macrophages, microglia, oligodendrocytes, osteoclasts, platelets, testis (Gutman et al. 2005).

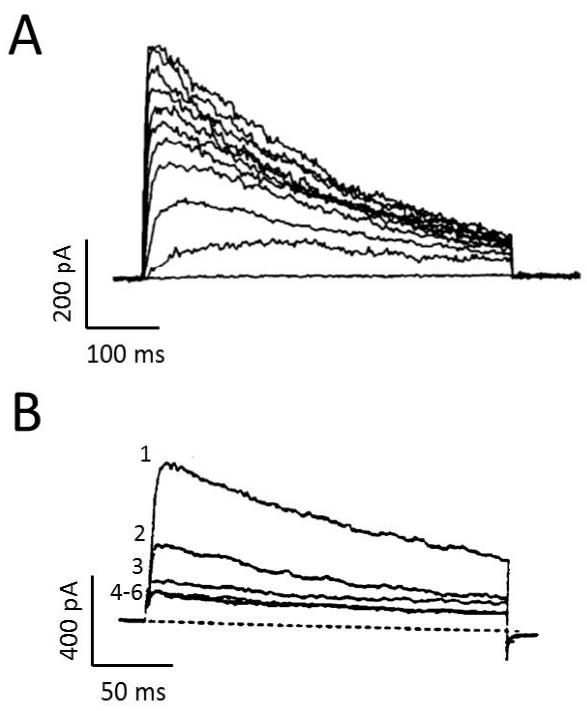

Figure 9. Biophysical characteristics of $K_v1.3$ channel. (A) K^+ currents in an outside-out patch configuration from an oocyte injected with $K_v1.3$ mRNA. The membrane potential was -80 mV, and depolarizing pulses were applied every 45 s. The test potential was changed from -50 to 50 mV in 10-mV increments. (B) Cumulative inactivation of $K_v1.3$ currents obtained from a human peripheral blood lymphocyte. From Grissmer et al. 1990

$K_v1.3$ biosynthesis begins in the ribosomes of the rough endoplasmic reticulum (Kanda and Abbott 2012). Like the rest of K_v channels, $K_v1.3$ lacks the ERR signal that directs mRNA translation product directly to the ER, so they are thought to use the S2 segment to target the protein to ER. In addition, S1 transmembrane domain determines channel topology. In the interior of the ER some post-translational modifications such as glycosylation are performed before reaching the Golgi network, where N-linked glycosylation in the S1-S2 extracellular loop would be achieved (Spencer et al. 1997). It is also in the ER, while the nascent proteins are actively being translated from the ribosome, where the tetramerization of the channel through the N-terminal T1-recognition domain is generated. In vitro studies suggest that the assembly of the tetramers occurs before membrane insertion and by means of a

dimerization of dimer subunits (Tu and Deutsch 2004). There is an intense debate and controversy about where K_v channels heterotetramerize or associate with auxiliary subunits. For most transmembrane proteins multimerization occurs in the ER because proper folding of protein subunits produces the required topology for specific quaternary contacts, and is often a prerequisite for efficient export of these proteins from the ER. Some groups suggest that the same N-terminal tetramerization domain (T1) allows different α-subunits to form complexes. Others propose that gene expression levels modulate the probability of heteromeric formation (Weiser et al. 1994). Evidence in T cells proposed that K_v channel diversity and proportion of homomeric and heteromeric complexes is determined by the degree of temporal overlap and kinetics of expression instead of the spatial segregation of monomeric pools (Panyi and Deutsch 1996).

The $K_v1.3$ channel N-terminus domain mainly contains the T1-tetramerization domain and, together with the C-terminus domain, it also includes several potential phosphorylation sites for PKA, PKC, tyrosine kinases as well as for CaMKII activity. This phosphorylation activity regulates cell signalling, channel surface expression and membrane potential (Cahalan and Chandy 1997). $K_v1.3$ sequence includes also a di-acidic motif in its C-terminus which is responsible to interact with COP-II proteins and mediate the forward trafficking to the plasma membrane (Martínez-Mármol et al. 2013). The last residues of the C-terminus of the channel correspond to a PDZ-domain responsible to anchor the channel to special domains of a cell, such as the immunological synapse in T cells or in spines in neurons (Matkó 2003).

$K_v1.3$ knockout mice are known as "super-smellers" because they have a 1000-10000-fold lower threshold for the detection of odours and an increased ability to discriminate between odorants. $K_v1.3^{-/-}$ mice produce not only a higher number of synaptic glomeruli in olfactory bulbs, but also a significant increase in the levels of various signalling molecules, such as the neurotrophic tyrosine receptor kinase B (TRKB), the cellular kinase Src, the adaptor proteins 14-3-3, nShc, PSD-95 and growth factor receptor-bound protein (GRB) (Fadool et al. 2004). The body weight of the Kv1.3 gene-targeted mice was less than that of the wild-type mice (Xu et al. 2003). Initially, $K_v1.3$-deficient mice showed no abnormalities neither in the total amount of lymphocytes nor in the proportion of types in thymus, spleen, and lymph nodes. Also, no differences in thymocyte apoptosis or T cell proliferation were detected respect wild-type, in spite of a significant increase in compensatory chloride currents in $K_v1.3^{-/-}$ thymocytes (Koni et al. 2003). However, further research in immune cells from $K_v1.3^{-/-}$ mice revealed interesting results.

1.1.5. Voltage-dependent potassium channel $K_v1.5$

$K_v1.5$ channel was cloned originally from heart (Roberds and Tamkun 1991). $K_v1.5$ is encoded by the intronless kcna5 gene, which is located in the chromosome 12p13.3. It produces a 613 aminoacid protein in human that encodes the pore-forming α subunit of a delayed rectifier K_v channel. When expressed as a homomultimer in a heterologous system (Gutman et al. 2005), its pore conductance has a typical value of 8 pS. The filter selectivity only allows the flow of potassium ions. Kv1.5 is a voltage-dependent channel, which opens at -14 mV, inactivates very slowly ($\tau > 5$ s) and lacks cumulative and C-type inactivation. Its steady-state inactivation value h_∞ is -25 to -10 mV. Although some general inhibitors, no specific and widely used blocker has been discovered.

$K_v1.5$, mainly a cardiac channel, is the responsible for the ultrarapid-activating K^+ current in heart (IK_{ur}). It is also expressed in aorta, colon, kidney, pooled colon, kidney, stomach, smooth muscle,

whole embryo, hippocampus and cortex (oligodendrocytes, microglia, Schwann cells), pituitary and pulmonary artery (Gutman et al. 2005).

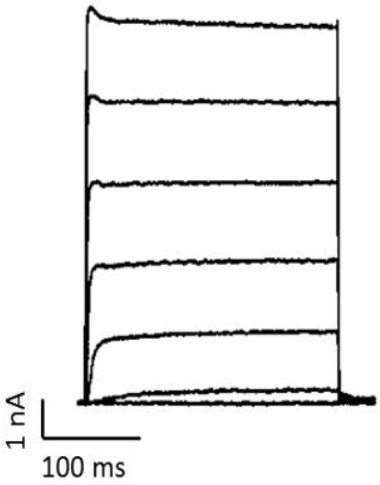

Figure 10. Outward potassium currents in HEK 293 cells expressing EGFP-K_v1.5. Currents were elicited by step depolarizations (-60 to +60 mV) from an initial holding potential of -80 mV. From Remillard et al. 2007.

This channel suffers posttranslational modifications such as N-glycosilation or palmitoylation and interacts with Src kinases, and gets modulated by PKA and PKC kinases (Jindal et al. 2008; David et al. 2012). K_v1.5 coassembles with K_v1.3 in heterologous system or phagocytes or with K_v1.2 in VSCM (Albarwani et al. 2003; Vicente et al. 2006), thus forming functional heterotetrameric complexes which stoichiometry determines trafficking properties (Vicente et al. 2008). K_v1.5 possess a basic intracellular motif (RR) near the T1 tetramerization domain that serve as a ER retention signal, which explains the retained phenotype of this channel in the ER when expressed in heterologous system (Colley et al. 2007). K_v1.5 forms also macromolecular complexes with auxiliary subunits such as $K_v\beta$s and KCNEs (Pongs and Schwarz 2010), modifying trafficking or biophysical properties, and with the scaffolding protein SAP97 and caveolin-3, regulating lipid raft distribution (Folco et al. 2004).

Experiments of K_v1.5 overexpression in COS, HEK-293 and PASMC (pulmonary artery smooth muscle cells) have been reported to cause membrane hyperpolarization and increase cell shrinkage associated to apoptosis. Furthermore, the downregulation of the channel by means of short interfering RNA caused membrane depolarization and increased cytosolic [Ca^{2+}], while pharmacological agents that blocking of K_v1.5 by using pharmacological tools (4-AP and others) stimulated PASMC contraction, migration, and proliferation, but inhibited apoptosis (Remillard et al. 2007).

1.1.6. Pharmacology of K_v1.3 and K_v1.5 channels.

Ion channels are important drug targets because they play a crucial role in controlling a very wide spectrum of physiological processes and because their dysfunction can lead to pathophysiology. Ion channels have a rich and diverse pharmacology (Hille 2001).

Molecules that block K_v1.3 channels belong to two large classes: small molecule blockers and peptide toxins. Small molecule blockers, such as 4-AP (Fig. 11A), TAE or Psora-4 (Fig. 11B), typically have a small molecular weight, are hydrophobic and thus easily permeate through cell membranes. In the water-filled cavity located at the middle of the channel they bind from the intracellular side to the internal end of the selectivity filter. Due to their smaller size these molecules have fewer contact points with the channel than peptide toxins, which generally results in lower affinity and poorer selectivity. In contrast, K_v1.3-blocking peptide toxins from scorpions, snakes, sea anemones and marine cone snails typically consist on short peptides (<70 residues) that are stabilized by multiple disulphide bonds. They carry a large positive net charge and consequently are non-permeant through cell membranes. They interact with the external end of the pore, thus impairing the efflux of K^+, and they bound through

a large interacting surface with the channel. Some examples of these toxins are MgTX (Fig. 11C) from the scorpion *Centruroides margaritatus* or ShK from the sea anemone *Stichodactyla helianthus* (Varga et al. 2010).

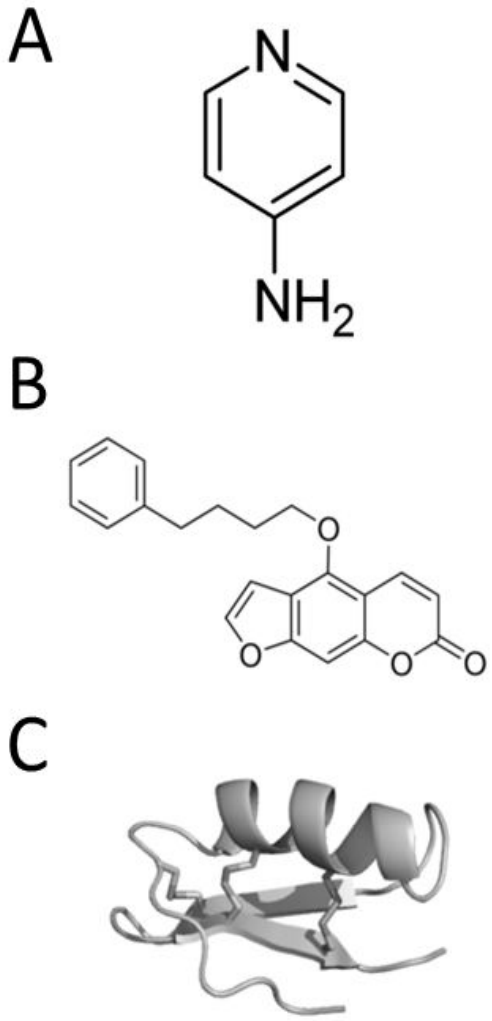

Figure 11. K_v1.3 inhibitor structures. (A) 4-aminopyridine, (B) Psora-4 and (C) Margatoxin.

The K^+ channel classical blockers 4-AP and TEA block Shaker channels. The typical and historical K_v channel blocker TEA (tetraethylammonium) is capable of blocking the channel from both sides, although it is membrane non-permeant. External TEA interacts with a tyrosine or a histidine at the carboxyl-terminal end of the P-region. Another classic and widely used blocker, 4-AP, acts selectively and reversibly by increasing the apparent rate of inactivation, suggesting that it blocks the open channel from the cytosolic side. In addition, 4-AP seemed to be trapped in the closed state (Grissmer et al. 1994; Varga et al. 2010; Judge and Bever 2006). Ampyra™ (dalfampiridine) is the name of the oral derivative drug of 4-AP approved by the FDA in 2010 to improve walking ability in people with multiple sclerosis (FDA Administration 2010). Although 4-AP is a potent convulsant with narrow toxic-to-therapeutic ratio, its beneficial effects could arise not only from blockade of CNS synaptic channels but also from effects on microglia and/or T cells (Judge and Bever 2006).

A potent small-molecule inhibitor of K_v1.3 channels is the 5-(4-Phenylbutoxy)psoralen (Psora-4). The template 5-methoxypsoralene was initially extracted from *Ruta graveolens*, a plant that had been reported to have beneficial effects in multiple sclerosis. Although the precise binding of the compound into the K_v1.3 is still unknown, Psora-4 is a membrane-permeant compound that blocks the K_v1.3 channel in a use-dependent manner with a Hill coefficient of 2 and an EC_{50} value of 3 nM, by preferentially binding to the C-type inactivated state of the channel. It exhibits 17-70-fold selectivity for Kv1.3 channels over closely related K_v1 family channels (K_v1.1, K_v1.2, K_v1.4 and K_v1.7), with the exception of K_v1.5 channels (EC_{50} = 7.7 nM). In a test of *in vivo* toxicity in rats, Psora-4 did not display any signs of acute toxicity after five daily subcutaneous injections at 33 mg/kg body weight. Psora-4 selectively suppressed the proliferation of human and rat myelin-specific effector memory T cells with EC_{50} values of 25 and 60 nM, respectively, without persistently suppressing peripheral blood naive and central memory T cells. The limited selectivity of Psora4 for K_v1.3 channels over the cardiac channel K_v1.5 channel impairs the use as a useful therapeutic. In spite of that, by introducing an additional oxygen atom into the side-chain linker of Psora-4, PAP-1 was generated and it inhibited K_v1.3 in a more potent manner (IC_{50} =

2nM) and displayed 23-fold selectivity over $K_v1.5$ (Vennekamp et al. 2004; Triggle et al. 2006). Administration of PAP-1 suppressed delayed-type hypersensitivity in rats indicating its potentiality as an oral available immunomodulator. In a standard model for type-1 diabetes mellitus (T1DM), the $K_v1.3$ specific inhibitor-treated PAP-1 group showed a reduction in the development of experimental autoimmune diabetes (EAD) of a 50% with respect to the control group due to a decreased infiltration of T cells and macrophages in the pancreatic islets and to a reduced β cell destruction (Schmitz et al. 2005). Also, in a rat model of allergic contact dermatitis (ACD) a PAP-1 derivative potently suppressed inflammation by inhibiting the infiltration of $CD8^+$ T cells and reducing the production of the inflammatory cytokines IFN-γ, IL-2, and IL-17. Interestingly, even topical application of PAP-1 was as effective as intraperitoneal or oral administration, which opens the possibility of developing a drug for the topical treatment of inflammatory diseases such as psoriasis (Nguyen et al. 2012).

Margatoxin was isolated from the scorpium *Centruroides margaritatus* and it is a membrane-non-permeant inhibitor (Garcia-Calvo et al. 1993; Leanza, Henry, et al. 2012). This inhibitor occludes the pore like a cork plugs a bottle. They stabilize this interaction by extending a critical lysine (position 28 in MgTX) into the pore and the rest of the peptide structure makes several secondary contacts with the surface of the channel (Triggle et al. 2006). It was in 1997 when the first in vivo study was performed showing that MgTX inhibited a delayed-type hypersensitivity and the antibody response to an allogenic challenge (unfortunately, it also presented a reduction of overall thymic cell population and an inhibition of the correct thymic development of T cell subsets) (Koo et al. 1997). The most potent $K_v1.3$ peptide inhibitor is ShK. This peptide from the Caribbean Sea anemone *Stichodactyla helianthus*, blocks the channel under physiological conditions with a K_d value of 11 pM. Several pharmacological studies on animal models are being performed to assess its potentiality as an immunomodulator. ShK-186, a Shk derivative, effectively treated disease in a rat model of multiple sclerosis with no toxicity. In rat models of rheumatoid arthritis and allergic asthma, the $K_v1.3$ inhibitor ShK derivative significantly ameliorated the affected joints during the 21 days of the treatment than the control group of animals or inhibited the activation and proliferation of peripheral blood T cells as well as reduced the total immune infiltrates of lymphocytes, eosinophils and neutrophils in the bronchoalveolar area, respectively (Chandy et al. 2004; Matheu et al. 2007; Beeton et al. 2006; Varga et al. 2010; Koshy et al. 2014). This inhibitor has been deeply studied pharmacologically in rats and monkeys (Tarcha et al. 2012) and it started a first-in-man phase-1 trial (Chi et al. 2012).

The use of high affinity and specificity $K_v1.3$ inhibitors in *in vivo* treatment and prevention experiments turned out to be very safe, the amount of the compound need for therapeutic use does not cause cardiac toxicity assessed by continuous electrocardiogram (EKG) monitoring and it does not alter clinical chemistry and haematological parameters after a 2-week therapy. Several parameters characterizing the pharmacokinetics of $K_v1.3$ inhibitors were determined and improved to increase stability. The relative safety of $K_v1.3$ blockers may be due in part to channel redundancy or compensation by other channels and also because $K_v1.3$ blockers may not inhibit $K_v1.3$-containing heteromultimers with the same affinity as $K_v1.3$ homotetramers in T cells (Varga et al. 2010; Beeton et al. 2006; Rangaraju et al. 2009; Felipe et al. 2006; Villalonga et al. 2007).

$K_v1.5$ is also inhibited by the general potassium channel blockers 4-AP and TEA, and also by the potent chemical inhibitor Psora-4, which blocks both $K_v1.3$ and $K_v1.5$. $K_v1.5$ is highly insensitive to $K_v1.3$ blockers (Table 1) and has no known specific pharmacology. However, new chemicals such as

S0100176 or diphenyl phosphine oxide-1 (DPO-1) that potently inhibit $K_v1.5$ have been discovered (Comes et al. 2013).

	4-AP K_d, [μM]	MgTX IC_{50}, [pM]	Psora-4 EC_{50}, [nM]
$K_v1.1$	290	10000	62 ± 9
$K_v1.2$	590	520	49 ± 6
$K_v1.3$	195	110	2.9 ± 0.3
$K_v1.4$	13	---	202 ± 12
Kv1.5	270	No effect	7.7 ± 0.9
$K_v1.6$	1500	50000	---
$K_v1.7$	150	No effect	100 ± 12
$K_v3.1$	29	No effect	1500 ± 300
HERG	No effect	No effect	5000 ± 500
$K_{Ca}3.1$	No effect	No effect	5000 ± 400
SKCa3	No effect	No effect	5000 ± 500
BK	No effect	No effect	5000 ± 200
$Na_v1.2$	No effect	No effect	1400 ± 100

Table 1. K_d, Ic_{50} and Ec_{50} values of 4-aminopyridin, margatoxin and Psora-4 respectively, on cloned ion channels. Adapted from Vennekamp et al. 2004; Gutman et al. 2005; Rangaraju et al. 2009

1.1.7. Accessory proteins: KCNEs

Regulatory subunits, often termed as auxiliary subunits, of voltage gated channels are non-pore forming structures that modify expression, subcellullar localization, trafficking or functional properties of the channel complex. The topology of these proteins varies widely within the voltage-gated superfamily (Pongs and Schwarz 2010). (i) $K_v\beta$ subunits do not span the membrane and interact with the N-terminal T1 domain of K_v channels. The N-terminus tail of $K_v\beta$ cytosolic proteins from vertebrates functions as an inactivating gate for K_v1 α subunits, by entering the pore and blocking the K^+ efflux during sustained channel opening. (ii) The KChIp1-4 (K^+ channel interacting proteins) are another family of β auxiliary subunits and they provide Ca^{2+}-sensitivity to K_v4 channels due to its Ca^{2+} sensing domain. They interact with K_v4 channels enhancing their expression and modulating their properties by binding to the intracellular T1 domain. (iii) The MinK related regulatory subunits or KCNEs are a five closely related protein family with a single transmembrane segment with both a small extracellular and intracellular domain. (iv) KChAPs (K^+ Channel Associated Proteins) act as chaperones for Kv2.1 and Kv4.3 channels, binding also to $K_v1.x$ channels but, with the exception of $K_v1.3$, not increasing their currents (Kuryshev et al. 2001). (v) DPPLs (Dipeptidyl aminopeptidase-like proteins) are transmembrane proteins with a short N-terminus cytoplasmic domain known to interact with K_v4 channels. (vi) Other proteins like AKAP, calmodulin, etc.

KCNE protein subunits are encoded by the *kcne* gene family and consist of 103 to 170 residues (<20 kDa), with a single TM domain and some glycosylation sites in the extracellular N-terminus domain. These peptides associate with α subunits to form stable assemblies with unique properties and different from α subunits alone. The family of peptides encoded by the KCNE genes has five known members. The founding member, MinK for "minimal potassium channel", is encoded by *kcne1* and was discovered in 1988 by functional expression of fractionated rat kidney RNA. Injection of its cRNA in *Xenopus laevis* oocytes was found to surprisingly generate a slow activating K^+ current, although its short length. Time later, $K_v7.1$ α subunits were found to be present endogenously in those cells, so their association increased the current density and explained the phenomena. A decade later, *kcne2*, *kcne3*, *kcne4* and, finally, *kcne5* were discovered by focusing on MinK critical motifs using homology-based searches of expressed sequence tag databases. The corresponding gene products were designated as MinK-Related Peptides (MiRPs), and then as KCNEs. As evidenced by their role in health and in several inherited diseases, these subunits appear to be important modulators of the channelosome and cell physiology (Abbott and Goldstein 2001; Solé and Felipe 2010; Lundquist et al. 2006). The most well-

known and described interactions occur with K_v7.1, although they can also interact and modulate other K_v channels and assemble with other KCNEs or with calmodulin (Ciampa et al. 2011).

1.1.7.1. KCNE1

The slow component of the delayed rectifier current (I_{Ks}) is essential for normal cardiac repolarization, and it is generated by the interaction of KCNE1 with KCNQ1. KCNE1 slows the activation kinetics of the channel, and it also alters the inactivation or the conductance of the complex. It may interact also with HERG and Kv4.3. It is a 129 residues peptide encoded by two different transcripts: *kcne1a* and *kcne1b*, the latter of them seems to be cardiac-specific. The inheritance of single missense mutations in KCNE1 (and also in KCNQ1) leads to a disease called Long QT Syndrome (LQTS). This disease, so named because the appearance of an EKG is a prolongation of the QT interval, is characterized by a heart rhythm condition that can potentially cause fast and chaotic heartbeats, thus triggering a sudden seizure and, in some cases, sudden death. Individuals who carry two mutant alleles of KCNE1 can suffer also congenital deafness (Jervell and Lange-Nielsen syndrome) because endolymph production is disrupted in the cochlea. *kcne1* transcripts are found in heart and auditory epithelium, but also in kidney, eye, gastrointestinal tract, and T cells. The analysis of Kcne1$^{-/-}$ mice showed a disrupted K^+ secretion and degeneration of the inner ear, although presenting minor changes in cardiovascular function (Lundquist et al. 2006; Abbott and Goldstein 2001). Regarding the immune system, it is important to point out that KCNE1 and K_v1.3 were simultaneously cloned in human Jurkat T cells (Attali et al. 1992). *Kcne1* gene expression was also found in peripheral blood leukocytes although it was not detected in spleen or thymus (Chouabe et al. 1997).

1.1.7.2. KCNE2

KCNE2, originally named MiRP1, is a 123 residues protein that can easily reach the plasma membrane, but when coexpressed with specific α subunits, it prevents their anterograde trafficking to Golgi, their cell polarization trafficking and distribution, their biophysical properties or mediate their internalization. KCNE2 alters activation and confers constitutive activity when coexpressed with KCNQ1, decreasing its current magnitude. It can also interact with HERG channels leading to the cardiac I_{kr} current and may modulate hyperpolarization-activated cation channel-4, K_v4.2, and K_v4.3. In murine ventricles KCNE2 interacts with K_v1.5 and controls the localization of the channel in the intercalated discs (Roepke et al. 2008). An interaction between KCNE2 and K_v1.3 channels has been proposed in the choroid plexus epithelium, by keeping the voltage dependence of the channel but decreasing its current density. That way, these complexes would allow anion efflux in the CSF but would prevent hypokalorrachia (abnormally low [K^+] in the cerebrospinal fluid) (Roepke et al. 2011). Mutations in this peptide generate inherited cardiac arrhythmias, consistent with its involvement in repolarization of the myocardium. It is noteworthy that some polymorphisms in the *kcne2* gene increase the susceptibility to acquire arrhythmia in response to certain drugs. The Kcne2$^{-/-}$ mouse exhibits cardiac arrhythmia and hypertrophy, achlorhydria, gastric neoplasia, hypothyroidism, alopecia, stunted growth and choroid plexus epithelia dysfunction, illustrating the depth of the influence of KCNE2. Screening studies suggest that *kcne2* gene is expressed at very low levels in several tissues, including leukocytes, spleen and thymus (Lundquist et al. 2006; Abbott and Goldstein 2001; Abbott 2012).

1.1.7.3. KCNE3

KCNE3, or MiRP2, is a 103 residues protein widely expressed in skeletal muscle, rather than in cardiac muscle. It alters activation and confers constitutive activity when coexpressed with KCNQ1, increasing its current magnitude. KCNE3 can also modulate HERG and $K_v3.4$. The interaction with this channel is thought to contribute to the skeletal muscle resting membrane potential and, due to the rapid recovery from inactivation of the complex, it facilitates the rapid trains of action potentials. Mutations of the KCNE3 gene lead to muscle affections such as familial periodic paralyses. *kcne3* gene disruption in mice disrupts intestinal chloride secretion, predisposes to ventricular arrhythmogenesis and also impairs auditory function. According some studies, KCNE3 is the most highly expressed *kcne* gene across various tissues and it is expressed in leukocytes, thymus and spleen (Lundquist et al. 2006), although some other analysis report an absent expression of RNA in leukocytes (Abbott et al. 2001).

1.1.7.4. KCNE4

KCNE4, a 170 aminoacid protein originally named as MiRP3, is encoded by 4 exons within the *kcne4* gene localized in chromosome 2q36.1. A single site of transcription initiation has been identified in human ventricular mRNA for KCNE4. Several transcription factors are thought to be involved in *kcne4* promotor such as MyoD, a transcription factor that plays a major role in regulating muscle differentiation, or E4F1, another transcription repressor that functions in cell survival and proliferation through control of the cell cycle. Northern blot analysis revealed that KCNE4 is strongly expressed in heart, skeletal muscle and uterus, less in kidney, lung and placenta, and weakly in liver, spleen, testes, ovarian tissue, small intestine, brain and blood cells. Mouse KCNE4 was cloned by Grunnet et al. (2002), whereas Teng et al. (2003) cloned the human KCNE4 from heart tissue. The human KCNE4 protein sequence shows 90 % homology to mouse KCNE4 and 38 % to KCNE1 (Teng et al. 2003; Grunnet et al. 2003; Lundquist et al. 2006; Grunnet et al. 2002).

KCNE4 functionally regulates $K_v7.1$, $K_v4.2$, $K_{Ca}1.1$, $K_v1.1$ and $K_v1.3$, in heterologous coexpression experiments. KCNE4 inhibits $K_v7.1$ activity, but a single nucleotide polymorphism in the cytoplasmic end of KCNE4 generating an E145D substitution, increases $K_v7.1$ current and it is an independent risk factor for atrial fibrillation in Han and Uygur Chinese (Ma et al. 2007). KCNE4 regulates potassium urinary excretion through its interaction with $K_{Ca}1.1$, which results in a current reduction (Levy et al. 2008). In murine cardiac myocytes, KCNE4 contributes to cardiac transient outward potassium currents (I_{to}) through a ternary complex between $K_v4.2$, KChIP2 and itself (Levy et al. 2010). Actually, *kcne4* gene deletion reduced K_v currents in male mouse atrial myocytes, by >45 % (Crump et al. 2015). KCNE4 also inhibits heterologously expressed $K_v1.1$ and $K_v1.3$ channels but have no effects on a variety of other voltage-gated potassium channels ($K_v1.2$, $K_v1.4$, $K_v1.5$, and $K_v4.3$). However, a recent study suggests that KCNE4 forms complexes with $K_v1.5$ increasing both its surface expression in CHO cells and its current magnitude (Crump et al. 2015). Although biophysical affection occurred on both $K_v1.1$ and $K_v1.3$ when coexpressed with KCNE4, surface expression is not affecting the first one (Lundquist et al. 2006). Moreover, studies from our laboratory indicate that KCNE4 alters and functions as an inhibitory $K_v1.3$ subunit. KCNE4 decreases current density, slows activation, accelerates cumulative inactivation, retains the channel in the ER (Fig. 12) and impairs its targeting to lipid raft microdomains (Solé et al. 2009). It has been recently reported that KCNE4 can also interact with calmodulin through a tetraleucine motif and that may explain its role in heart tissue (Ciampa et al. 2011). In addition, a

genetic association study connects *kcne4* gene with allergic rhinitis disease (Freidin et al. 2013).

The KCNE4$^{-/-}$ mice model described a marked reduction of conscious heart rate and a short QT interval with possible implications for arrhythmia susceptibility, although the systolic blood pressure was similar to wild type animals. Knockout mice also showed a decrease in body temperature and an increase in blood phosphorous and calcium levels. Unlike body weight and length, blood levels of albumin, alkaline phosphatase, alanine aminotransferase, blood urea nitrogen, creatinine, triglycerides or uric acid, KCNE4$^{-/-}$ mice presented a trend towards reduced blood glucose and increased cholesterol levels. Regarding the immune system, no differences were found in the hematology profile or in the number of CD4^{+} cells, CD8^{+} cells, NK cells, B cells or monocytes. The acute phase response (TNFα, MCP-1, or IL-6 blood levels) or the ability to produce IgG_1 or IgG_2 antibodies following antigen challenge remaned als unaltered (Ciampa 2011).

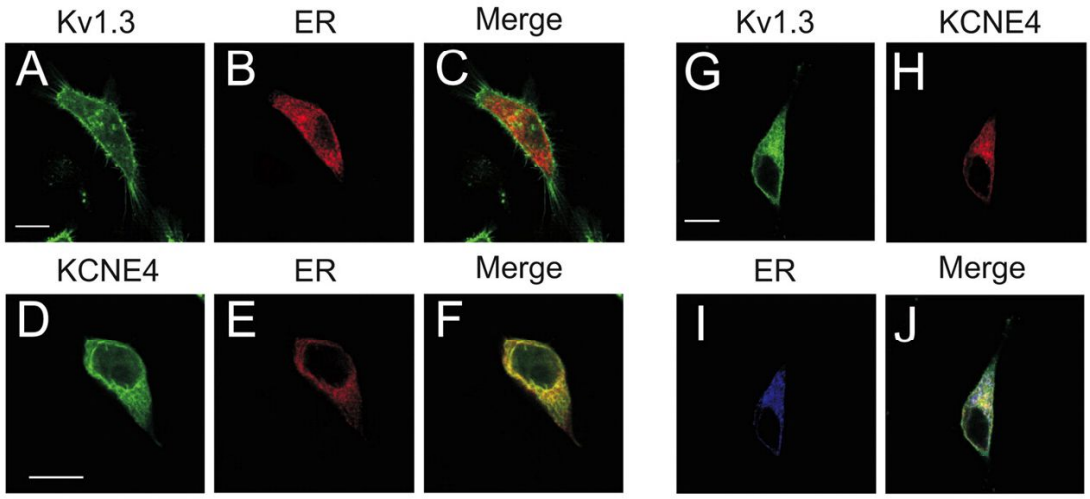

Figure 12. KCNE4 alters K_v1.3 membrane expression. YFP-K_v1.3 and KCNE4-CFP have distinct cellular distributions in transiently transfected HEK293 cells with YFP-K_v1.3, KCNE4-CFP, YFP-K_v1.3/KCNE4-CFP and DsRed-ER marker. (A-C) K_v1.3 does not colocalize with the ER. (D-F) KCNE4 shows strong ER colocalization. (G-J) KCNE4 retains K_v1.3 at the endoplasmic reticulum. Coexpression of K_v1.3. Yellow indicates colocalization. White colour indicates triple colocalization. Scale bars: 10 µm. From Solé et al. 2009.

1.1.7.5. KCNE5

KCNE5 or MiRP4, a 142 residues protein encoded by the intronless *kcne5* gene in the X chromosome, was found in a gene-rich segment of DNA, the deletion of which results in a syndrome denoted as AMME for Alport syndrome (an X-linked gene deletion syndrome characterized by glomerulonephritis and sensorineural hearing loss), mental retardation, midface hypoplasia, and elliptocytosis. KCNE5 is only known to interact biophysically with KCNQ1 in heterologous systems. A screening study observed high levels of KCNE5 expression in brain, thymus, testes, spleen, ovaries, and placenta, where KCNQ1 was not always present, suggesting potential interaction with other α subunits (Lundquist et al. 2006; Abbott and Goldstein 2001).

1.2. Immunitary system.

1.2.1. Generalities

The immune system is concerned on how the body is able to accept its own tissue as self, and to react against infectious agents or alien tissue from everybody else as non-self (Davis 2013). The immune function is divided into innate and adaptive immunity. The former represents a rapid response to a large but limited number of stimuli whereas the latter elaborates a specific response to each specific antigen and generates also memory. Mast cells, phagocytes, basophils, eosinophils and natural killer cells belong to the innate immunity while B and T lymphocytes to the adaptive immunity. T cells are thymus-derived and, as effector cells, orchestrate cell-mediated immunity and eliminate pathogens by means of helper, cytotoxic or regulatory functions. They divide vigorously upon antigen stimulation but, once the pathogen is cleared, they become apoptotic and die. In contrast, B lymphocytes differentiate into antibody-secreting plasma cells and mediate the humoral immune responses. Long-term protection against repetitive exposure to pathogens is provided by memory B and T cells that persist in the body in the absence of antigen after a primary infection. Those cells are able to survive, self-renew and rapidly expand after re-exposure to antigen. Memory responses are typically more rapid, have a greater magnitude and comprise antibodies of greater affinity (Seder and Ahmed 2003; Abbas, Lichtman, and Pillai 2007; Cruvinel et al. 2010).

The activation and proliferation of leukocytes are modulated by membrane transduction of extracellular signals. Changes in membrane potential are among the earliest events occurring upon stimulation of macrophages, and for more than 30 years, it has been widely accepted that Ca^{2+} influx is essential to trigger lymphocyte activation. In this context, K^+ channels indirectly determine the driving force for Ca^{2+} entry and they also form a functional and signalling network in lymphocytes at molecular levels (Cahalan and Chandy 2009; Varga, Hajdu, and Panyi 2010). $K_v1.3$ plays a major role in lymphocytes and phagocytes, such as macrophages and dendritic cells, which they also express $K_v1.5$. They both, $K_v1.3$ and $K_v1.5$, control leukocyte physiological responses (Vicente et al. 2003; Vicente et al. 2006; Villalonga et al. 2010; Villalonga et al. 2010; Zsiros et al. 2009). Moreover, $K_v1.3$ and $K_v1.5$ channels have been detected in several tissues during early stages of human development and, interestingly, abundant expression was found in fetal liver, which serves as a hematopoietic tissue during early gestation (Bielanska et al. 2010). Finally, several pharmacological studies that strategically target and selectively immunosuppress $K_v1.3$ channels for chronic inflammatory and autoimmune disorders are being developed due to their potential therapeutic benefits.

1.2.2. Macrophages and dendritic cells

Phagocytes are generated in the bone marrow (BM) as monoblasts and then evolve to monocytes which constitute 3-8 % of blood circulating leukocytes. When they reach connective tissues or parenchyma of organs, where they can remain months or years, phagocytes differentiate and generate macrophages and myeloid dendritic cells. Recently, the existence of three subpopulations of macrophages was proposed: activated, tissue repair, and regulatory macrophages (Mosser and Edwards 2009). Macrophages have an important role during inflammation, in innate immunity. By releasing pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, TNF-α, and chemokines, they increase vascular permeability and vasodilatation, thus attracting inflammatory cells, such as neutrophils, in the affected area. They also produce reactive oxygen species (ROS; such as O_2^-, OH^-, H_2O_2) and reactive nitrogen intermediates

like nitric oxide (NO). NO is produced by inducible nitric oxide synthase (iNOS), absent in resting macrophages, but induced by TLRs activation (Toll-like receptors), especially in the presence of INF-γ, which stimulates antigen presentation. During chronic inflammation, phagocytes express coestimulatory molecules and act as APCs, initiating the adaptive response (Cruvinel et al. 2010; Abbas et al. 2007). Macrophages express several potassium channels. $K_v1.3$, together with $K_v1.5$, may form heterotetrameric channels with different stoichiometry in mononuclear phagocytes adjusting cellular responses such as proliferation, apoptosis or differentiation (Vicente et al. 2006; Villalonga et al. 2007; Zsiros et al. 2009; Leanza et al. 2012). LPS and TNF-α regulate macrophage activation by up-regulating $K_v1.3$ and down-regulating $K_{ir}2.1$ expression (Vicente et al. 2003).

Dendritic cells are the most efficient phagocytes. They become activated by engulfing pathogens and uptaking antigens and, consequently, DCs migrate to regional lymph nodes, where they process and present on their surface protein or lipid antigens bound to MHC class II molecules to T cells. Immature DCs are extremely efficient in capturing antigens, while mature DCs are very efficient in presenting antigens (Cruvinel et al. 2010; Abbas et al. 2007). $K_v1.3$ expression is increased during dendritic cell maturation, accompanied by a down-regulation of $Na_v1.7$ channel, and the predominant outward K^+ current in mature dendritic cells strongly correlates biophysically and pharmacologically with $K_v1.3$ (Zsiros et al. 2009). $K_v1.3$ and $K_v1.5$ channels are coexpressed in cells of the phagocytic lineage (Vicente et al. 2003), including mouse dendritic cells (Matzner et al. 2008) and blood-derived dendritic cells in the central nervous system (Mullen et al. 2006). It is noteworthy to mention that gene deletion of $K_v1.3$ in DCs does not impair their ability to present Ag and activate T cells (Gocke et al. 2012).

1.2.3. B lymphocytes.

B lymphocytes are produced initially in the vitelline sac, in the liver during foetal life and, finally, in the BM. The cells that will differentiate into B cells remain in the BM during the maturation process, where TdT, RAG1 and RAG2 proteins will command the gene recombination necessary for the production of immunoglobulins (Ig). In the process of negative selection, still in the BM, immature B cells which recognize self-antigens with high affinity, undergo apoptosis or initiate a process called receptor edition, in which autoreactive Igs are substituted to produce mature B cells which will leave the BM. Then, mature B cells leave the BM and enter the circulation, migrating to the secondary lymphoid organs following chemoattractant gradients like CXCL13 or BCA-1, which are needed for B cell homing to follicles in the lymph node (Ansel et al. 2000). B cells are responsible for the humoral immunity, characterized by the production and secretion of antibodies capable of neutralizing or destroying the antigens against which they were generated. In order to do so, B cells must be activated, which results in a process of proliferation and differentiation that culminates in the generation of plasma cells with the production of Igs with high-affinity for the antigen epitope that originated the response. The B cell receptor (BCR), which is composed by a membrane-bound antibody flanked by Igα/Igβ heterodimers, drives B cell development, differentiation, proliferation and survival by transducing signals through the Igα/Igβ heterodimer cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) domains (Mesquita Júnior et al. 2010; Abbas et al. 2007).

Evidence suggests that mature naive B cells express K_v and K_{Ca} currents with properties resembling those of $K_v1.3$ and $K_{Ca}3.1$. LPS-activated B cells present high K_v currents and nonspecific K^+ channel inhibitors suggest a functional role for these channels in B cell mitogenesis (Amigorena et al. 1990). Memory B

cells, distinguished by the expression of the B cell maturation marker IgD and the member of the TNFR family CD27 required for generation and long-term maintenance, are a major source of pathogenic IgG autoantibodies that contribute to tissue damage in multiple sclerosis, T1DM and rheumatoid arthritis. Because of that, some research revealed that $K_v1.3$ and $K_{Ca}3.1$ channels are expressed in B cells, although only the latter suffer upregulation upon activation (Wulff et al. 2004).

1.2.4. T lymphocytes.

T lymphocytes represent approximately the 30 % of white blood cells. All T cells originate from lymphoblastic progenitor cells in the BM. Then, thymocytes or pre-T cells ($CD4^-CD8^-$) enter the thymus through the arteries and, during the differentiation process, they migrate towards the BM again, from where they go into the circulation. The maturation process involves the expression of a functional T cell receptor (TCR) and the co-receptors CD4 or CD8. The TCR consists of a membrane-bound αβ heterodimer (TCRαβ), four CD3 chains and a ζ-chain homodimer. The TCRαβ dimer recognizes antigenic peptides, while the associated signalling chains transduce signals with their cytoplasmic ITAM domains. About 98 % of thymocytes die during the development processes in the thymus by failing either positive or negative selection. During positive selection in the thymus cortex, self-antigens are presented to T cells through MHC and, if $CD4^+/CD8^+$ thymocytes are capable of interact and bind to MHC II class or MHC I class (neither too strongly nor too weakly), they will survive and differentiate into $CD4^+$ or $CD8^+$ T cells, respectively. Then, in the thymic medulla, negative selection consists on erasing T cells that bind too strongly to MHC molecules containing self-antigens, thus avoiding self-reactive T cells and autoimmune diseases (Mesquita Júnior et al. 2010; Abbas et al. 2007; Friedl et al. 2005).

Lymphocytes express predominantly two potassium channels, the voltage-gated $K_v1.3$ and the calcium-activated $K_{Ca}3.1$ potassium channels (also known as IKCa1; encoded by the *KCNN4* gene). $K_v1.3$ and $K_{Ca}3.1$ channels are similar regarding the conductance properties (both channels are highly K^+ selective and have similar single-channel conductance in the order of 10–14 pS). However they are remarkably different in their gating (Fig. 13) and blocker sensitivity (Table 1). While $K_v1.3$ is activated by membrane depolarization, $K_{Ca}3.1$ is a type of intermediate-conductance Ca^{2+}-activated K^+ channel activated by the increase in the cytosolic free Ca^{2+} concentration, and they are not deactivated even after prolonged exposure to increased cytosolic Ca^{2+} concentration. The elevation of intracellular Ca^{2+} that occurs in the first few seconds after TCR binding, opens the $K_{Ca}3.1$ channel, whose Ca^{2+} activation threshold is approximately 200 nM due to the permanently associated calmodulin by means of the C-terminus of the channel. The consequent K^+ currents make the membrane potential more negative and hyperpolarized (Panyi et al. 2014; Panyi et al. 2004; Rangaraju et al. 2009).

Besides $K_v1.3$ and $K_{Ca}3.1$, some T-cells also express $K_v1.1$, $K_v1.2$, and $K_v1.6$ (Liu et al. 2002). Although in $CD8^+$ and double negative mouse T cells $K_v3.1$ channel is responsible for K_v currents, the presence of this channel in human T cells opens debate (Grissmer et al. 1992; Rangaraju et al. 2009). In addition, while Jurkat cells express $K_{Ca}2.2$ and TRESK (Pottosin et al. 2008), human peripheral blood T cells express also TASK1 and TASK3, which contribute to membrane potential (Panyi et al. 2014; Rangaraju et al. 2009).

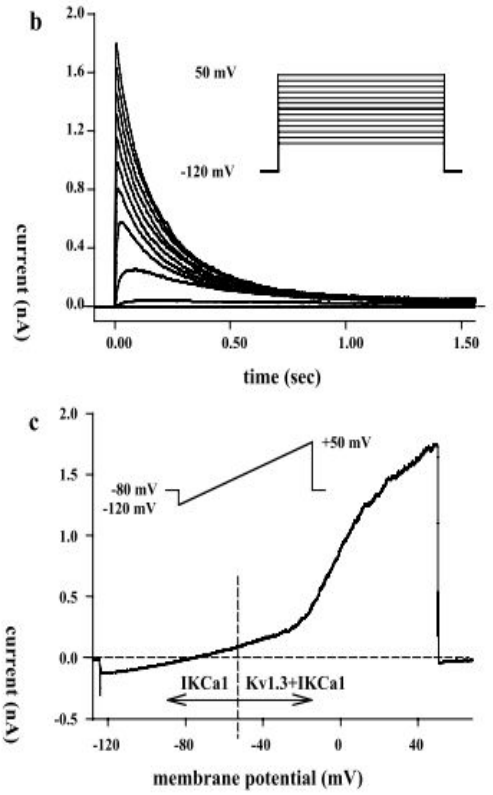

Figure 13. Basic biophysical properties of T cells. Maybe also from (Rangaraju et al. 2009)

Fully mature human T lymphocytes can be separated into three subpopulations based on their phosphatase CD45RA surface expression and the chemokine receptor required for homing to lymph nodes CCR7: naïve T cells, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Naïve T cells express both markers and are $CD45RA^{+}CCR7^{+}$. Long-lived T_{CM} lymphocytes, the largest pool of circulating memory T cells which retain the memory of a specific antigen, maintain the expression of CCR7 so they are able to recirculate through secondary lymphoid organs but lose the expression of CDRA45 and therefore are $CD45RA^{-}CCR7^{+}$. In lymph nodes, they transform into T_{CM}-effectors after antigen encounter and then migrate to inflamed tissue where, after a repeated antigenic stimulation arising from a chronic infection or autoimmune disease, can be induced to differentiate into T_{EM} cells This third population of T_{EM} lymphocytes, which quickly migrate to inflamed sites and secrete large volumes of cytokines to perform immediate effector functions and represent fewer than 20 % of circulating T cells, are negative for the expression of either marker ($CD45RA^{-}CCR7^{-}$). As shown in Fig. 14, quiescent naïve, T_{CM} and T_{EM} lymphocytes express 200-300 $K_v1.3$ and 5-35 $K_{Ca}3.1$ channels per cell (Varga et al. 2009; Wulff et al. 2003). However, activation with either an antigen or a mitogen induces drastic changes in potassium channel expression in the T cells subpopulations, producing different phenotypes. While naïve or T_{CM} subsets overexpress $K_{Ca}3.1$ channels and keep $K_v1.3$ low, T_{EM} cells upregulate $K_v1.3$ channel expression to 1500 channels/cell with little or no change in $K_{Ca}3.1$ levels. As a consequence, the inhibition of either $K_v1.3$ or $K_{Ca}3.1$ by the appropriate selective blockers can inhibit cell proliferation and function in a T cell subtype-specific manner. T_{EM} cells activated at sites of inflammation produce cytokines including IFNγ, IL-4 and IL-5 (Panyi et al. 2014).

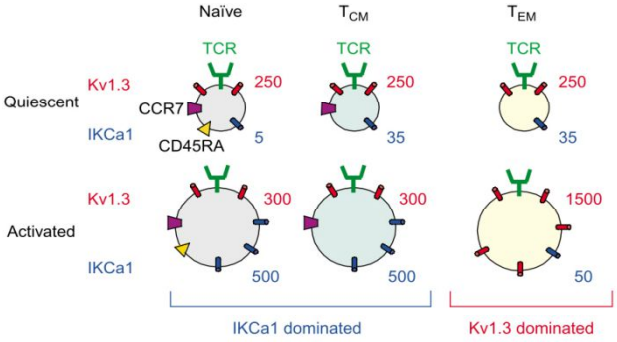

Figure 14. Naïve and T_{CM} cells increase IKCa1 channel expression following activation, whereas T_{EM} cells increase $K_v1.3$ channel expression. The average numbers of $K_v1.3$ channels and IKCa1 channels per cell in naïve ($CCR7^{+}CD45RA^{+}$), central memory (T_{CM}) ($CCR7^{+}CD45RA^{-}$) and effector memory (T_{EM}) ($CCR7^{-}CD45RA^{-}$) cells. From Chandy et al. 2004.

T cell membrane is highly resistant (10-20 GΩ), thereby the membrane potential can be regulated by a small number of $K_v1.3$ channels (Panyi 2005). In basal T cells, the resting potential is maintained at about -50 mV by only a small fraction of the available $K_v1.3$ channels, most of them in the inactivated state (Cahalan et al. 1985; Oortgiesen 1995; Varga et al. 2010). Transfection of the $K_v1.3$

channel gene alone into CHO cells was sufficient to shift the resting membrane potential to −50 mV approximately, as compared to the −5 to −20 mV membrane potential measured in non-transfected cells (Defarias et al. 1995). In addition, this membrane potential dependence of $K_v1.3$ regulates the cytolytic granules secretion and killing capability of some NK T cells, which destroy foreign cells (Sidell et al. 1986).

In T cells, $K_v1.3$ is part of a signalling channelosome on top of controlling membrane potential, thus reinforcing the idea of non-conduction function associated to ion channels (Kaczmarek 2006). This complex (Fig. 15) includes β1 integrin, PSD-95, SAP-97 (or hDlg), a signalling protein zeta-interacting protein (ZIP or sequestosome 1/p62) and the tyrosine kinase $p56^{Lck}$. $K_v1.3$ is also associated with $K_v\beta2$ subunit, the most abundant $K_v\beta$ subunit in human T cells, which is also upregulated during T cell activation and may act as a chaperone, accelerating the formation and stabilizing the structure of the channel complex (Autieri et al. 1997; McCormack et al. 1999; Shi et al. 1996; Gong et al. 1999; Cahalan and Chandy 1997). This channelosome provides a mechanism to physically link $K_v1.3$ channels through their N- and C-termini to β1-integrin, PKCζ regulation, cytoskeletal elements, and via Lck to CD4 and the TCR. This signalosome couples external stimuli through the TCR complex and β1 integrin with intracellular signalling cascades (Rangaraju et al. 2009; Tóth et al. 2009; Varga et al. 2010).

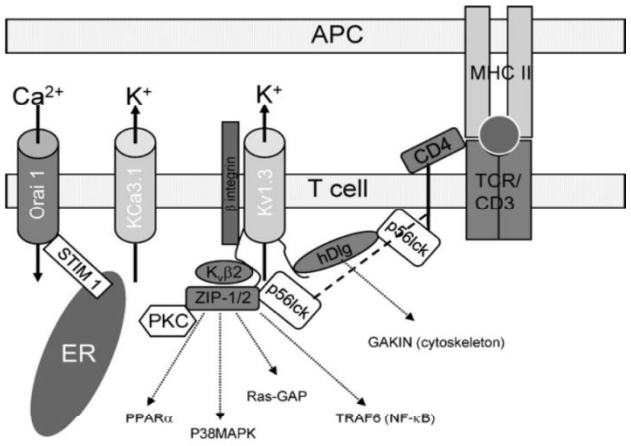

Figure 15. Ion channels and $K_v1.3$-associated proteins in the immunological synapse (IS). From Varga et al. 2010.

1.2.5. Immunological synapse and lymphocyte activation.

T lymphocyte activation, and therefore, the initiation of the adaptive immune response, is initiated by the physical contact with an antigen presenting cell (APC) such as macrophages, dendritic or B cells. The generation of an efficient immune response requires the clonal expansion of lymphocytes recognizing a given antigen specifically. Class II major histocompatibility complex proteins (MHC II) of APCs loaded with processed antigens co-assembled with the T cell receptor/CD3 complex (TCR/CD3) of T cells (Grakoui et al. 1999). The immunological synapse (IS) is a cell polarization structure in which certain plasma membrane and cytosolic proteins and intracellular organelles accumulate at the area of the T cell - APC contact forming a highly organized signalling zone. On the T lymphocyte side of the immunological synapse, the characteristic organization called supramolecular activation complex (SMAC) can be divided into two concentrically organized subcomplexes: a central supramolecular activation complex (c-SMAC) enriched in TCR complexes and PKC θ, and a peripheral supramolecular activation complex (p-SMAC) where integrin LFA-1 or ICAM-1 are

present (Krummel and Cahalan 2010; Abbas, Lichtman, and Pillai 2007).

Within seconds of TCR complex and coreceptors clustering within membrane lipid rafts and antigen recognition through MHC-peptide engagement, the TCR initiates a tyrosine phosphorylation cascade through LcK kinase that triggers multiple branching signalling pathways. Phosphorylation of ITAMS allows further kinases to be recruited and activated by different phosphorylation cascades, such as ZAP-70. An important cascade derived from those initial TCR-mediated signals is the activation of the Ras-MAP kinase pathway, which contributes directly to the synthesis and activation of transcription factors such as AP-1. Another important pathway derived specifically from increased tyrosine kinase activity is the activation of phospholipase C γ (PLCγ). This enzyme, located close to the membrane by SH2 domains and LAT (Linker for Activation of T cells) association, cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP_3). At this point the two additional signalling pathways of lymphocyte activation diverge. DAG activates the protein kinase C (PKC-θ) pathway, and, in turn, this kinase phosphorylates several intracellular substrates and inhibitors leading to the activation of another transcription factor, NFκB, thereby triggering specific gene transcription, such as cytokines or their receptors. The second pathway, initiated by the generation of IP_3, evokes a biphasic increase in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). This Ca^{2+} signal activates the Ca^{2+}-calmodulin-dependent phosphatase calcineurin, which dephosphorylates the transcription factor NFAT (nuclear factor of activated T cells), and consequently allowing it to accumulate in the nucleus and bind to the promoter element of IL-2 gene and others. Therefore, leading to cytokine production and ending with T cell proliferation (Panyi 2005; Rangaraju et al. 2009; Abbas et al. 2007).

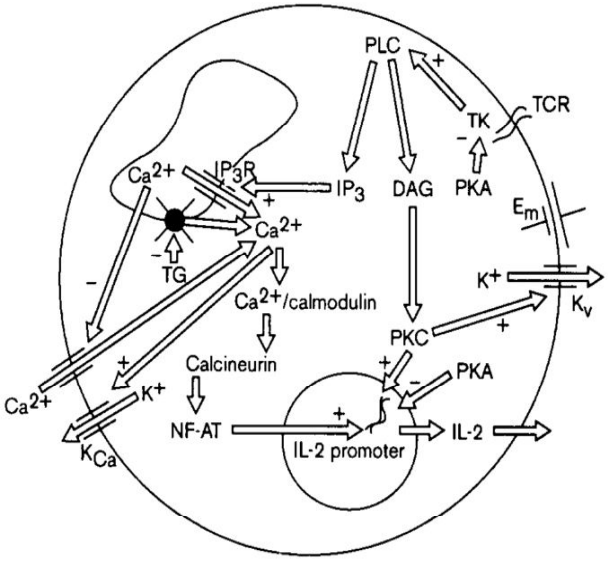

Figure 16. T cell activation pathways derived from TCR engagement to MHC. From Cahalan and Chandy 1997.

Calcium signalling is essential for lymphocytes to activate, secrete cytokines, migrate and proliferate (Rangaraju et al. 2009; Lewis 2001). During T cell activation, the TCR causes the generation of IP_3, which releases Ca^{2+} from the ER Ca^{2+} store to the cytosol through the IP_3 receptor. The stromal interaction molecule 1 (Stim1) monitors the ER Ca^{2+} store through EF-hand motifs and, upon depletion of the ER store, is activated, oligomerized and redistributed into discrete puncta located in junctional ER sites that are in close proximity to the plasma membrane, thereby activating extracellular Ca^{2+} influx through calcium-release activated calcium (CRAC) channels formed from Orai1 subunits. Actually, removal of extracellular Ca^{2+} and deletion or mutations of STIM1 or ORAI1 in human T cells inhibit the sustained Ca^{2+} influx and the T cell activation (Grissmer, Lewis, and Cahalan 1992; Panyi, Beeton, and Felipe 2014; Feske 2007; Cahalan et al. 2007). The influx of Ca^{2+} raises the $[Ca^{2+}]_i$ from a resting value of 50–100 nM into the low micromolar range. Once the CRAC channels are activated, the electrochemical driving force for Ca^{2+} will determine the magnitude of the inward current. The electrical driving force for Ca^{2+} entry through CRAC channels increases with membrane hyperpolarization and is determined by the

resting potential of T cells, which is maintained predominantly by $K_v1.3$ and $K_{Ca}3.1$ K^+ channels. The activation of these channels provide the counterbalancing K^+ efflux required for the maintenance of a negative membrane potential and that of the Ca^{2+} influx. Therefore, K^+ channels in T cells promote calcium entry and regulate amplitude and frequency of Ca^{2+} oscillations by modulating the membrane potential (Panyi et al. 2004; Rangaraju et al. 2009; Lewis 2001). CRAC channel (Krause and Welsh 1990), $K_v1.3$ (Chi et al. 2012; Tóth et al. 2009; Panyi et al. 2004) and $K_{Ca}3.1$ (Sallusto et al. 1999; Ghanshani et al. 2013) redistribute to the zone of contact at the immunological synapse (Fig. 17) and form a functional network sustaining the Ca^{2+} signal in T cells (Krummel and Cahalan 2010; Cahalan and Chandy 2009; Panyi, Beeton, and Felipe 2014; Chi et al. 2012).

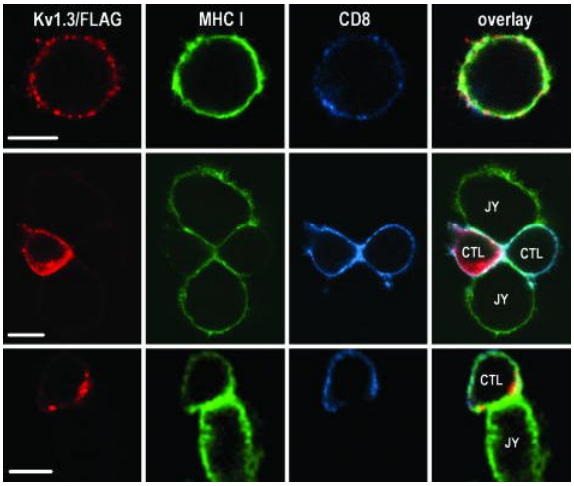

Figure 17. Enrichment of Kv1.3/FLAG in or around the IS. Distribution of Kv1.3/FLAG, MHC class I, and CD8 molecules in a lone CTL (top) and CTL-JY target cell conjugates (middle and bottom). In lone CTLs, the channels are distributed in small patches throughout the membrane (Top). On CTL-target interaction Kv1.3 channels are redistributed and enriched in the IS in the majority of CTLs (middle) or surround the synapse like a ring (bottom). Scale bar: 5 µm. From Panyi et al. 2004.

Upon activation, $K_v1.3$ channels increase in number even though there is no simultaneous increase in mRNA, suggesting that the regulation is posttranscriptional (Ghanshani et al. 2013). T cell activation is inhibited by blocking K^+ channels. Charybdotoxin (ChTX) and margatoxin (MgTX), which block $K_v1.3$ of peripheral lymphocytes, by means of plasma membrane depolarization (Sands, Lewis, and Cahalan 1989), inhibit TCR-mediated calcium influx (Verheugen and Korn 1997; Grinstein and Smith 1990; Grissmer, Lewis, and Cahalan 1992), IL-2 production (Freedman et al. 1992), and proliferation (Leonard et al. 1992). Several pharmacological studies report how $K_v1.3$ blocking leads to a failure TCR- or mitogen-dependent proliferation and to a defective production of many cytokines, including IL-2. This phenomenon is also physiologically relevant in the immunotolerance at the placenta, because its high levels of progesterone, which inhibits $K_v1.3$ and $K_{Ca}3.1$ channels, impair T cells activation (Ehring et al. 1998).

Gene deletion of $K_v1.3$ in mice, besides previous mentioned considerations, results in significantly decreased incidence and severity of experimental autoimmune encephalomyelitis (EAE). Antigenic stimulation of myelin oligodendrocyte glycoprotein (MOG)-specific $K_v1.3$-KO T_h cells *in vitro* results in a significant upregulation of IL-2Rα and CTLA-4 in association with an increase in pSTAT5, which is a kinase crucial for T_{reg} and T_{h2} differentiation. Whereas $K_v1.3$ KO T_h $CD4^+$ lymphocytes present in a limited proliferative capacity and secrete large amounts of IL-2 and IL-10 in vitro (Gocke et al. 2012; Grishkan et al. 2015), $CD4^+CD8^+$ thymocytes expressed mRNAs and/or proteins for several other K^+ channels, including $K_v1.1$, $K_v1.4$, Kv1.6, $K_v2.1$, $K_v3.1$, and $K_{Ca}2.2$, and they also exhibited a compensatory upregulation of anion currents with properties resembling ClC-3 (Koni et al. 2003).

Molecules of the IS are lipid raft associated and able to rearrange dynamically after the activation of TCR-CD3 complex (Tóth et al. 2009). Lipid rafts are membrane microdomains enriched in sphingolipids, glycosphingolipids, and cholesterol, and also in signal transduction enzymes and

components of the cytoskeleton that act as platforms for proteins lateral sorting and signalling (Dykstra et al. 2001). During T cell activation, $K_v1.3$ concentrates in the immunological synapse together with lipid rafts (Panyi et al. 2004). Cholesterol and protein-lipid interactions also regulate $K_v1.3$ gating, because the depletion or transformation of rafts into large ceramide-enriched membrane platforms inhibits channel activity (Bock et al. 2003; Hajdú et al. 2003). Polarized cell surface expression and localization of K_v channels is regulated in Jurkat T cells by PSD-95 and SAP97 which are adaptor proteins that interact through the PDZ-binding motif located in the C-terminus of $K_v1.3$. PSD-95, in particular, takes part in the recruitment of $K_v1.3$ into the IS, confirming its important role in human T-cell activation (Szilágyi et al. 2013). In T cells from patients with systemic lupus erythematosus, the defective temporal and spatial $K_v1.3$ distribution and trafficking to the IS may contribute to T cell abnormal functions (Nicolaou et al. 2007).

1.2.6. Autoimmune diseases

Nearly 80 different autoimmune diseases are known, and they affect 125 million people worldwide. Autoimmune diseases include various organs in the body such as the joints (rheumatoid arthritis), heart, lungs, central nervous system (multiple sclerosis), endocrine organs (type 1 diabetes mellitus) and skin (psoriasis). In these diseases, tissue destruction is mediated by autoreactive (self-reactive) immune cells. T cell negative selection is not 100 % effective, because some autoreactive T cells escape thymus censorship, and are released into the circulation. Additional mechanisms of peripheral tolerance active in the periphery exist to silence these cells such as anergy, deletion, and regulatory T cells. If these peripheral tolerance mechanisms also fail, autoimmunity may arise (Abbas et al. 2007; Chi et al. 2012).

The $K_v1.3$ channel is an interesting target for the treatment of diverse autoimmune diseases. Multiple sclerosis is a chronic degenerative disease of the central nervous system in which gradual destruction of myelin occurs in patches throughout the brain or spinal cord, interfering with nerves and causing muscular weakness, loss of coordination and speech and visual disturbances. The expression of $K_v1.3$ in post mortem multiple sclerosis brain tissue is shown to be high (Rus et al. 2005). Demyelinating lesions contain various immune cells that mediate the inflammatory and neurodegenerative processes: macrophages, microglia, DC and T cells all up-regulate $K_v1.3$ when fully activated, with microglia differentially expressing $K_v1.5$ at early stages of cellular activation and DC predominantly expressing $K_v1.5$ over $K_v1.3$ following activation (Judge and Bever 2006). In patients with multiple sclerosis, myelin antigen-specific patient T_{EM} cells were $CCR7^{-}$ and $K_v1.3^{high}$, whereas T cells specific for pancreatic antigens (insulin or GAD) from the same patients were $CCR7^{+}$ T cells and $K_v1.3^{low}$, similar to naïve T cells. In the perivenular infiltrate and parenchymal infiltrate of plaques in brains of patients with multiple sclerosis, T_{EM} cells are abundant and many of these cells stained positively for $K_v1.3$ (Rus et al. 2005). Again, in patients with rheumatoid arthritis, T_{EM} cells from the affected joints were $K_v1.3^{high}$, whereas T cells from non-autoimmune osteoarthritis patients were $K_v1.3^{low}$ (Chi et al. 2012; Beeton et al. 2006). Even in preliminary studies with psoriasis mouse models, the injection of the $K_v1.3$ blocker ShK showed a marked therapeutic effect (Gilhar et al. 2011).

1.3. Ion channels in the nervous system

Potassium channels are critical in determining the shape, the duration, and the frequency of action potential firing in excitable cells of the nervous system. Alterations in the function and specific subcellular localization of these channels (e.g. K_v1 channels are mainly found on axons and nerve terminals) generate perturbations in membrane excitability and neuronal function. Mutations in K^+ channels are linked to neuronal disorders such as episodic ataxia and benign familial neonatal convulsions (Vacher et al. 2008).

Unlike $K_v1.1$, $K_v1.2$ and $K_v1.4$, highly expressed in brain, the other Shaker channels have a relative lower expression (Vacher, Mohapatra, and Trimmer 2008). However, $K_v1.3$ is highly expressed in the cerebellar cortex (Veh et al. 1995; Kues and Wunder 1992). Strong immunostaining and ^{125}I-margatoxin binding were found in the molecular layer of the parallel fiber axons of cerebellar granule cells, which also contained high levels of staining for $K_v1.1$, suggesting that $K_v1.1$ and $K_v1.3$ may form heteromeric channels on parallel fibers. In addition to the rat cerebral cortex, the $K_v1.3$ channel is also expressed at particularly high levels in the mouse auditory brainstem (Gazula et al. 2010) and in mitral cells of the olfactory bulb (Vacher et al. 2008; Fadool et al. 2004). As previously mentioned, $K_v1.3^{-/-}$ mice, alternatively named "super-smellers", are highly able to discriminate between odorants and produce a higher number of synaptic glomeruli in olfactory bulbs. $K_v1.3$ has also been detected in the inferior colliculus, the pons/medulla, the midbrain, the superior colliculus, the corpus striatum and the hippocampus (Gutman et al. 2005). One feature of $K_v1.3$ that distinguishes it from other channels in the K_v1 family is that it undergoes cumulative inactivation with repeated depolarization. In general, this property widens the action potentials diversity and the increased excitability as a neuron is repetitively stimulated (Kaczmarek 2006).

In general, $K_v1.5$ expression in the brain is quite low, mainly restricted to non-neuronal cells. For example, $K_v1.5$ and $K_v1.3$ are components of delayed rectifier currents in glia and endothelial cells. It is noteworthy that microglia, the central nervous system endogenous immune cells, express $K_v1.3$, IKCa1 and there are some evidences for $K_v1.5$ expression (Kotecha et al. 1999). This differentiated macrophage proliferates, migrates to sites of injury, presents antigen, phagocytizes, secretes proinflammatory cytokines and cytotoxins, and generates a respiratory burst producing cytotoxic reactive oxygen and nitrogen species. $K_v1.3$ regulates microglia proliferation and both channels, $K_v1.3$ and IKCa1, are involved in the respiratory burst (Fordyce et al. 2005). Furthermore, during development, $K_v1.3$ localizes to the central and peripheral nervous systems, while $K_v1.5$ overlaps mostly with the central nervous system (Bielanska et al. 2010).

1.4. Cancer

1.4.1. Generalities and types of cancer

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Some of the tumoral characteristics, summarized in Fig. 19, include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, inducing genome instability and mutations, reprogramming cellular metabolism, and evading immunological destruction while promoting inflammation at the same time (Hanahan and Weinberg 2011). There are more than 100 types of cancer that affect humans, classified according to the location in the body where the cancer first developed or to the

type of tissue in which the cancer originates (histological type), such as carcinomas, sarcomas, myelomas, leukemias, lymphomas and mixed (U. S. National Institutes of Health 2015; Hanahan and Weinberg 2011). Due to the immense complexity of cancer pathology, only the types of neoplasms that are studied in this thesis are going to be mentioned.

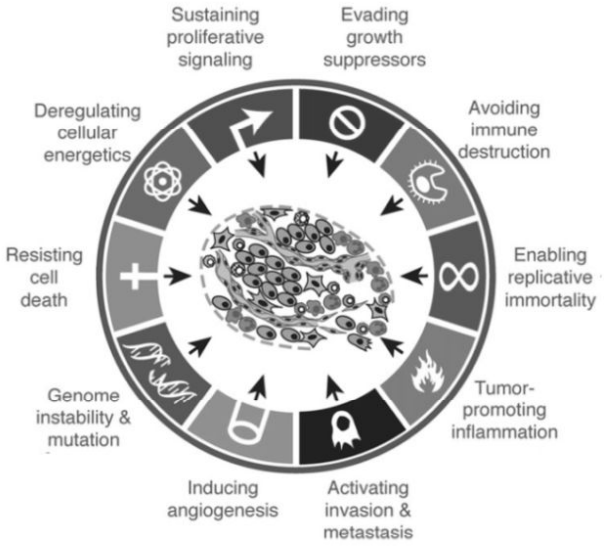

Figure 18. The Hallmarks of cancer. From Hanahan and Weinberg 2011.

Lymphoma is a type of cancer that begins in cells of the lymph system. The two main types of lymphoma are i) Hodgkin lymphoma, which is the most common and often cured type of lymphoma characterized by the presence of large and abnormal lymphocytes in the lymph nodes called Reed-Sternberg cells, and ii) non-Hodgkin lymphoma (NHL), which includes many different types that form from different types of white blood cells (usually B cells, but also T cells and NK cells). The most common types of NHL in adults are diffuse large B-cell lymphoma, which is usually aggressive, and follicular lymphoma, which is usually indolent (U. S. National Institutes of Health 2015).

Gliomas are considered brain tumours because they start and arise from brain or spinal cord glial cells, which are non-neuronal cells that maintain homeostasis, form myelin and provide support and protection for neurons. Gliomas represent the 30 % of brain tumours and they are classified according to their grade or pathologic evaluation status: low- (benign) and high-grade (malign) or according to the type of glial cell involved in the tumour: astrocytoma, ependymoma, oligodendroglioma or mixed. Glioblastoma, also known as glioblastoma multiforme (GBM) or grade IV astrocytoma is a type of astrocytoma rarely curable (length of survival of 12 to 15 months following diagnosis) (Young et al. 2015). No glioblastoma patient has been cured to date because of the bad prognosis, specially associated with gliomas of astrocytic origin, and the diffuse nature of glioblastoma cell invasion into the brain parenchyma and the natural resistance of glioblastoma migrating cells to apoptosis and, thus, to chemotherapy and radiotherapy (Le Calvé et al. 2010; Louis 2006).

Pancreatic ductal adenocarcinoma or PDAC is a malign neoplasm originally from transformed cells that come from pancreatic cancer. The most common type of pancreatic cancer, around 95 % of those tumours, is the adenocarcinoma coming from the exocrine part of the pancreas. A minority comes from the Langerhans island cells, and they are classified as neuroendocrine tumours. Signs and symptoms that will finally allow the diagnosis depend on the localization, size, type of tumour tissue and they can include abdominal pain, low back pain and jaundice. PDAC is the most lethal common cancer because it is usually diagnosed at an advanced stage and is resistant to therapy. This cancer has an extremely poor prognosis, especially for patients with metastasis (80 % of all cases) where the surviving tax is 15 % and the relative survival is from 6 to 10 months. Men and also African people or descendants are more prone to suffer from pancreatic cancer (Ryan et al. 2014).

1.4.2. Ion channels and proliferation

During the cell growth, the cell cycle starts with the G_1 (gap 1) phase, which separates the previous cell division from S-phase, when new DNA is synthesized. After DNA synthesis, the process is followed by the G_2 (gap 2), and then the mitotic phase (M). After mitosis, a cell can re-enter the cell cycle in G_1 phase, or enter a quiescent state (G_0). Progression through the cell cycle is regulated by control mechanisms known as cell cycle checkpoints, which have evolved to ensure proper timing of cellular events. Expression of different Cyclin/Cyclin-dependent kinases (Cyclin-Cdk) complexes changes during cell cycle to ensure sequential progression through its phases. Thus checkpoints are constitutive feedback control pathways, safeguarding the cell cycle transitions: G1/S, G2/M and the exit from mitosis. In addition, kinase activity of Cyclin-Cdk complexes is regulated by Cdk inhibitors, such as p27 or p21, which stop cell cycle progression under unfavourable conditions. Uncontrolled cell division or propagation of damaged DNA can contribute to genomic instability and tumorigenesis (Lim and Kaldis 2013; Alberts et al. 2002). Membrane potential has also been reported to act as a cellular bioelectric parameter that controls the progression through the cell cycle, influencing both G_1/S phase and G_2/M phase transitions (Blackiston et al. 2013). Several studies demonstrate that V_m is not constant during the cell cycle (during the G_1/S phase of cell cycle cells undergo a transient hyperpolarization). Cell types with a very hyperpolarized resting potential, such as muscle cells and neurons, typically showing little or no mitotic activity, are on average more depolarized than non-tumour cells. In this context, induced depolarization underwent DNA synthesis and mitosis in neurons (Urrego et al. 2014; Pardo 2004).

Experimental evidence demonstrates that inhibition of K^+ channel function with general blockers such as 4-AP or TEA leads to a decrease in proliferation both in models in which proliferation is a physiological response (T and B lymphocytes, brown fat cells, Schwann cells, neuroendocrine cells, hepatocytes, GH3 pituitary cells, endothelial cells, keratinocytes, corneal epithelium, retinal pigment cells, chondrocytes) and in those in which it is a manifestation of a pathological condition (melanoma, breast carcinoma, neuroblastoma, small-cell lung cancer, bladder cancer, myeloblastic leukemia, prostate cancer, hepatocarcinoma, mesothelioma, and colon cancer cells) (Pardo 2004; Wonderlin and Strobl 1996). Many drugs and toxins that specifically block the activity of K_v channels decrease cell proliferation, mainly during the G_1/S transition (Felipe et al. 2006).

K^+ channels influence cell cycle progression through permeation-related and non-canonical mechanisms. Even in plants, a high [K^+] increases the turgor pressure required for cell growth. By contrast, mitosis requires a transient decrease in turgor pressure allowing a K^+ efflux (Sano et al. 2007). K^+ channels also provide the driving force required for Ca^{2+} to enter the cell or for Na^+-dependent nutrient transport by changing the membrane potential to more negative values, and influencing the intracellular pH. Ca^{2+} is an important second messenger of intracellular signals. K^+ channels regulate cell volume, often in combination with Cl^- channels, and the involvement in volume regulatory responses, influences in turn proliferation, as well as migration, hormone release and gene expression (Hoffmann et al. 2009). Also of note, one study reported how K^+ channel blockers decreased proliferation of glioma cells while increasing their cell volume. Furthermore, cells exhibited their highest proliferation rates within a relatively narrow range of cell volumes, with decreased proliferation both over and under this optimal range (Rouzaire-Dubois and Dubois 1998). Membrane depolarization caused by inhibiting K^+ currents provoked also the accumulation of the

cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p21^{cip-1}$ (Ghiani et al. 1999; Villalonga et al. 2008). Also permeation-independent mechanisms that could involve protein–protein interactions between the channels and important effectors in proliferation such as 14-3-3, Src, or TNF-α receptor with $K_v11.1$, or β-integrins and p56lck with $K_v1.3$ or calmodulin with $K_v10.1$, may regulate cell proliferation. K^+ channel expression or function can itself be also regulated by progression through the cell cycle (Urrego et al. 2014; Pardo 2004; Kaczmarek 2006; Roura-Ferrer et al. 2008).

$K_v1.3$ was the first K_v channel to be linked with proliferation in T lymphocytes (DeCoursey et al. 1985). $K_v1.3$ blockade suppress T cell activation and Ca^{2+} signalling in human T cells. $K_v1.3$ has also controls the cell cycle in many other cell types, such microglia cells (Fordyce et al. 2005; Kotecha and Schlichter 1999), proliferating oligodendrocyte progenitors during G_1/S transition (Chittajallu et al. 2002), macrophages (Villalonga et al. 2010; Villalonga et al. 2007; Vicente et al. 2003). $K_v1.3$ functional expression is associated with a proliferative phenotype, because the blockade of the channel induces a significant inhibition of cell proliferation and keeps the contractile function in several muscular and endothelial cells (Erdogan et al. 2005; Tian et al. 2013; Cheong et al. 2011). $K_v1.3$ increases and controls smooth muscle cell proliferation by voltage-dependent conformational changes of the channel, without needing ion conduction, by in turn activating intracellular signalling pathways (Cidad et al. 2012; Jiménez-Pérez et al. 2015). Aberrant $K_v1.3$ expression is found in several types of cancer such as gliomas (Preußat et al. 2003), prostate cancer (Fraser et al. 2003; Abdul and Hoosein 2002), colon cancer (Abdul and Hoosein 2002), breast cancer (Abdul et al. 2003), and melanoma cells (Artym and Petty 2002). In addition, it is important to mention the dual role of $K_v1.3$ promoting both, proliferation and apoptosis (Szabò et al. 2008).

On the other hand, $K_v1.5$ channel is also involved in the proliferation of oligodendrocytes (Attali et al. 1997), astrocytes (Macfarlane and Sontheimer 2000), hippocampal microglia (Kotecha and Schlichter 1999), macrophages (Villalonga et al. 2010) and myoblasts (Villalonga et al. 2008). In fact, $K_v1.5$ expression changes throughout cell cycle progression with maximum expression occurring during the G_1/S phase and increased expression has also been noted during myogenesis (Vigdor-Alboim et al., 1999).

1.4.3. Ion channels and apoptosis

Apoptosis is a tightly controlled process of cell death characterized by nuclear condensation, cell shrinkage, loose of membrane integrity and DNA fragmentation. Apoptosis is triggered by activation of death receptors be death-promoting molecules (extrinsic pathway) or mitochondrial damage (intrinsic pathway) (Kunzelmann 2005). Cell shrinkage or apoptotic volume decrease (AVD), is an apoptosis feature resulting from changes in intracellular ions, with the particular loss of intracellular potassium. Activation of K^+ channels during apoptosis is much more pronounced than during proliferation, causing a decrease in K^+ concentration and ionic strength. This reduction plays a critical role in both the downstream activation of the apoptotic machinery such as caspase activation, apoptosome formation and apoptotic nuclease activity and the proliferative activity because many of the growth and mitosis related enzymes require a minimal $[K^+]$. Several potassium channels have been described to be involved in cell death physiology, and direct addition of inhibitors such as TEA or quinine can protect cells from apoptosis. In addition, another important factor could be Ca^{2+} signalling. A steady Ca^{2+} increase appears to be needed for apoptotic enzymes activity, while proliferation was

associated with oscillatory Ca^{2+} rises (Bortner and Cidlowski 2014; Comes et al. 2013).

$K_v1.3$ protein has been detected in mitochondria of lymphocytes and several immortalized cell lines. However, the mechanism by which it reaches this organelle has not been deciphered (Szabò et al. 2005). The location on the inner mitochondrial membrane allows a direct interaction with Bax, a pro-apoptotic protein. Bax lysine 128 is thought to mimic $K_v1.3$ toxin inhibitors effect and induce apoptosis by blocking $mtK_v1.3$ porus (Fig. 19), thus hyperpolarizing mitochondrial inner membrane and, consequently, releasing cytochrome C due to the production of reactive oxygen species (Szabò et al. 2008). Membrane-permeable inhibitors of $K_v1.3$ such as Psora-4, PAP-1 and clofazimine, induce cell death by directly targeting the mitochondrial channel in multiple human and mouse cancer cell lines and efficiently induce apoptosis of chronic lymphocytic leukemia cells (Leanza, Henry, et al. 2012).

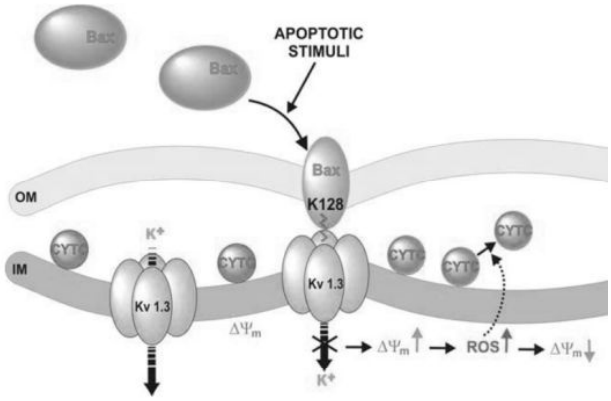

Figure 19. Model for action of mitochondrial $K_v1.3$ during apoptosis. From Szabò et al. 2008.

Because of low $[K^+]$ inhibitory effect on proliferative enzymes, $mtK_v1.3$ presence or both, K^+ channel inhibition or downregulation suppresses apoptosis in several cell types, including cancer (Remillard and Yuan 2004). A cancer cell would then evade apoptosis by simply downregulating potassium channels and, consequently, increasing cytosolic $[K^+]$. Interestingly, $K_v1.3$ expression is decreased in many types of human cancers while $K_v1.5$ mostly correlates with an increment (Comes et al. 2013; Bielanska et al. 2009), suggesting that this ion channel may help to apoptotic resistance. Currently, there is controversy in the field as to whether K^+ channels inversely correlate with apoptosis in cancer cells. Studies of viral infected and H-ras transformed cell lines with cancerous properties were reported to have enhanced K^+ channel activity (Teulon et al. 1992; Repp et al. 1993). In addition, siRNA down-regulation experiments of $K_v1.3$ and $K_v1.5$ in macrophages conferred them resistance to apoptosis (Leanza et al. 2012). Also, $K_v1.5$ channel expression was upregulated concomitantly to apoptosis induction in several cancer cell lines in experiments where mitochondrial membrane potential was inhibited by dichloroacetate (DCA) (Bonnet et al. 2007). However, K_v channel stimulation or overexpression might also result in a proapoptotic response by decreasing cytosolic ionic strength by an alternative mechanism (Bortner and Cidlowski 2014; Storey et al. 2003).

1.4.4. Ion channels and cancer

Highly proliferating cancer cells dysregulate and impair potassium channel expression in various cancer and tumour types. Potassium channels are involved in cell proliferation as they control cell cycle progression and show cell and tissue-specific expression. It has been suggested that a certain grade of malignancy correlates with the expression of potassium channels. Although several types of K^+ channels have been associated with cancer, such as $K_v1.1$, $K_v1.3$, $K_v1.5$, $K_v2.1$-9, $K_v3.4$, $K_v7.1$, $K_v10.1$-2, $K_v11.1$, $K_{ir}2.1$, $K_{ir}3.1$, $K_{ir}4.1$, $K_{Ca}1.1$, $K_{Ca}3.1$, $K_{2P}9.1$, TREK-1 and TASK.3, the list is not completed and the field deserves further research (Felipe et al. 2006; Li and Xiong 2011). If K^+ channel blocking impairs proliferation, the opposite effect should also be expected. Thus

cycle progression would also be accelerated by an increase in the K^+ channel expression levels. Actually, only introduction of K_v10.1 presented transforming activity or oncogenic effects, as long as an oncogene is a gene that has the potential to cause cancer when mutated or expressed at high levels (Pardo 2004; Pardo et al. 1999).

Several K_v channels are not only aberrantly expressed in tumours but also involved in their physiology. Several potassium channel blockers have shown anti-proliferative effects in tumour cells. K_v1.5 and, to some extent, K_v1.3 are aberrantly expressed in many human cancers. On the one hand, K_v1.3 is rarely upregulated in cancer cells. It is highly expressed in leukemia cells and moderately expressed in muscle sarcomas, colon cancer and breast cancer (although absent in normal tissue). Generally, K_v1.3 is absent or shows a low expression in stomach cancer, prostate cancer (decreasing respect the normal tissue), gliomas, kidney, lung, pancreas, ovary and skin cancer. On the other hand, K_v1.5 is generally expressed and mostly upregulated in several cancer types compared to normal tissues. Whereas this channel is highly expressed in skin cancer and present at moderate levels in colon cancer, gliomas, kidney and pancreatic cancer, it shows a lower expression in stomach, muscle, bladder and lung cancer. We can conclude that the abundance of K_v1.5 expression mostly increases in tumour cells, whereas K_v1.3 expression is generally downregulated (Comes et al. 2013).

2. AIM

2. AIM.

Voltage dependent potassium channels are a group of plasma membrane ion channels with a key role in regulating repolarization and resting membrane potential in electrically excitable cells. K_v channels also contribute to a wide variety of cellular processes including the maintenance of vascular smooth muscle tone, cell growth, adhesion, mobility, cell volume, epithelial transport, homeostasis, insulin release, and apoptosis. Increasing data implicate K_v channels in cell differentiation and cell cycle control. Non-specific drugs, such as 4-AP and TEA, lead to cell cycle arrest. Evidence suggests that K_v channels are remodelled during carcinogenesis.

K_v channels play a role in the immune system as the predominant ion channels controlling the resting membrane potential and tuning intracellular Ca^{2+} signalling in lymphocytes, monocytes, macrophages, and dendritic cells. A functional K_v channel is an oligomeric complex composed of pore-forming and ancillary subunits. The KCNE gene family (KCNE1-5) is a novel group of modulatory K_v channel elements expressed in several tissues including leukocytes. KCNE peptides are small single spanning membrane proteins known to modulate K_v channels trafficking and biophysical properties.

Leukocytes present a limited K_v repertoire, including $K_v1.3$ and $K_v1.5$ channel isoforms. $K_v1.3$ is expressed in the immune system, and the blockade of this channel is associated with selective inhibition of T cell activation and proliferation. In spite of its significance, the mechanisms that regulate $K_v1.3$ and its role in the T cell activation are not well known. In addition, recent data from our laboratory demonstrate that KCNE4, acting as a dominant negative ancillary subunit, physically interacts with $K_v1.3$ inhibiting K^+ currents and retaining the channel intracellularly.

Having all this in mind, the hypothesis of the present work was that changes in the channelosome composition by modulating the heterooligomeric combinations of the $K_v1.3$ channelosome control physiological and neoplastic cell growth as well as leukocyte responses.

Therefore, the specific objectives of the PhD dissertation are:

- To elucidate the role of $K_v1.3$ and $K_v1.5$ channels in cell growth and their relationship with cancer, in models such as lymphomas (non-Hodgkin lymphomas), pancreatic ductal adenocarcinoma (PDAC) and glioblastomas.

- To analyse the expression of KCNEs ancillary subunits upon different states of activation and proliferation of leukocytes (macrophages, T and B lymphocytes).

- To study the $K_v1.3$ modulation by the auxiliary subunit KCNE4 in leukocytes.

3. RESULTS

3.1. CHAPTER 1. $K_V1.3$ AND $K_V1.5$ IN CELL GROWTH AND NEOPLASIA.

3.1.1. Contribution 1.

Published in The Journal of Leukocyte Biology, 2013, Oct; 94(4):779-789.

Emerging role for the voltage-dependent K^+ channel Kv1.5 in B-lymphocyte physiology: expression associated with human lymphoma malignancy.

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SUMMARY

K_v channels, which play a role in the immune system, are remodelled during carcinogenesis. Leukocytes present a limited K_v repertoire, with $K_v1.3$ and $K_v1.5$ as isoforms that are involved in neoplastic processes, such as proliferation and migration. In this study, we identified $K_v1.5$ in B-lymphocytes, characterized its role in proliferation and migration, and analysed Kv1.3 and $K_v1.5$ expression in human non-Hodgkin lymphomas. $K_v1.3$ and $K_v1.5$ were found to be remodelled differentially; whereas $K_v1.3$ expression did not correlate with the state of dedifferentiation or the nature of lymphomatous cells, Kv1.5 abundance correlated inversely with clinical aggressiveness. Our data show that $K_v1.5$ is a determinant of human B cell proliferation and migration, thereby identifying this channel as a new target for immunomodulation. Our work also provides new insights into the use of $K_v1.3$ and $K_v1.5$ as potential targets during tumorigenesis.

Report of the PhD student participation in the article

Emerging role for the voltage-dependent K^+ channel $K_v1.5$ in B-lymphocyte physiology: expression associated with human lymphoma malignancy
Published in The Journal of Leukocyte Biology, Impact Factor (2013): 4.304

Albert Vallejo Gracia performed all the experiments and the data analysis of this article but the immunohistochemistry studies and the electrophysiological recordings.

Dr. Antonio Felipe Campo
Thesis director

JLB

Article

Emerging role for the voltage-dependent K^+ channel Kv1.5 in B-lymphocyte physiology: expression associated with human lymphoma malignancy

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RECEIVED FEBRUARY 21, 2013; REVISED JUNE 21, 2013; ACCEPTED JUNE 25, 2013. DOI: 10.1189/jlb.0213094

ABSTRACT

Kv, which play a role in the immune system, are remodeled during carcinogenesis. Leukocytes present a limited Kv repertoire, with Kv1.3 and Kv1.5 as isoforms that are involved in neoplastic processes, such as proliferation and migration. In this study, we identified Kv1.5 in B-lymphocytes, characterized its role in proliferation and migration, and analyzed Kv1.3 and Kv1.5 expression in human non-Hodgkin lymphomas. DLBCL, F, MCL, ALCL, and T, along with control N specimens, were analyzed. Kv1.3 and Kv1.5 were found to be remodeled differentially; whereas Kv1.3 expression did not correlate with the state of dedifferentiation or the nature of lymphomatous cells, Kv1.5 abundance correlated inversely with clinical aggressiveness. Whereas indolent F expressed noticeable levels of Kv1.5, aggressive DLBCL showed low Kv1.5 levels. In addition, control LNs expressed heterogeneous high levels of Kv1.3, which could indicate some reactivity, whereas Kv1.5 abundance was low and quite homogeneous. Our data show that Kv1.5 is a determinant of human B cell proliferation and migration, thereby identifying this channel as a new target for immunomodulation. Our work also provides new insights into the use of Kv1.3 and Kv1.5 as potential targets during tumorigenesis.

***J. Leukoc. Biol.* 94: 779–789; 2013.**

Abbreviations: 4-AP=4-aminopyridine, ALCL=anaplastic large cell lymphoma, ALK-1=activin receptor-like kinase 1, CTX=charybdotoxin, DLBCL=diffuse large B cell lymphoma, EMA=epithelial membrane antigen, F=follicular lymphoma, GC=germinal center, HEK=human embryonic kidney, Hscore=histoscore, Kv=voltage-dependent potassium channels, LTV=lentiviral, M=mitosis, MCL=mantle cell lymphoma, MgTx=margatoxin, MINECO=Ministerio de Economía y Competitividad, MUM-1=multiple myeloma oncogene-1, N=LN lymphoma, pA=peak amplitude, pF=capacitance values, Sc=scramble, shRNA=small hairpin RNA, T=T cell lymphoma, TB=tumoral B cell, TC=tumoral cell, WHO=World Health Organization

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Kv participate in the activation and proliferation of cells of the immune system [1]. Lymphocytes express the voltage-gated Kv1.3 channel that contributes to the resting membrane potential of the cells [1, 2]. This protein plays a critical role during activation and proliferation, and several studies point to this channel as an excellent target for immunomodulation [3]. Kv1.3 is expressed abundantly in T-effector memory lymphocytes. These T cells are key mediators in autoimmune inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, psoriasis, and type I diabetes, and Kv1.3-based therapies have proven effective in experimental models [3]. Although Kv1.3 plays a major role in lymphocytes, mononuclear phagocytes, such as macrophages and DCs, also express Kv1.5, and Kv1.3 and Kv1.5 control physiological responses [4–7]. Kv1.3 determines the level of macrophage activation, but this is conditioned by the presence of Kv1.5 [5]. In addition, Kv1.3 and Kv1.5 coassemble as heteromultimeric complexes, and the oligomeric stoichiometry compromises pharmacological responses [8, 9].

In B-lymphocytes, proliferation and activation rely on changes of membrane potential conducted by ion channels [10]. In T-lymphocytes, Kv1.3 is crucial for the production of IL-2 and progression through the cell cycle [1]. In mononuclear phagocytes, although Kv1.3 increases in proliferating microglia and controls macrophage growth [4, 11], Kv1.5 plays a

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crucial role during activation [6, 9]. Surprisingly, the role of Kv1.3 in B-lymphocytes is uncertain. Although Kv channel inhibitors impair DNA synthesis [12], Kv1.3 blockers do not halt proliferation in B cells [13, 14]. In this context, $[K^+]$-induced depolarization reduces the IL-2 production in T-lymphocytes [15], and similar membrane depolarization inhibits Kv1.5 without affecting other Kv channels in pituitary cells [16]. Therefore, other Kv channels, such as Kv1.5, may be present in B cells and could account for malignant growth and cell proliferation [17, 18].

As Kv1.3 and Kv1.5 are involved in cell growth [4, 19], their presence in tumor cells and their pathophysiological functions therein have also attracted great attention [20]. Thus, Kv1.3 and Kv1.5 expression has been studied in various types of cancers [20]. In most cases, a clear relationship between aberrant Kv channel expression and malignancy and invasiveness has not been observed [21]. This apparent discrepancy may be attributed to remodeling mechanisms that could depend on the tumorigenic state [20, 22]. In numerous myeloid and lymphoid immortalized cancerous cell lines, Kv1.3 and Kv1.5 participate in neoplastic processes, such as proliferation, migration, and invasion; therefore, it follows that they would also contribute to cancer development [20, 21].

The role of Kv1.3 in B-lymphocytes is controversial, and the function of Kv channels during neoplastic growth is unknown in B cells. Therefore, in the present study, we initially characterized the presence of Kv1.5 in human B cells and investigated its role during proliferation and migration. Next, we analyzed Kv1.3 and Kv1.5 channels in several human non-Hodgkin lymphomas. Unlike Kv1.3, Kv1.5 contributed to cell proliferation and modulated B cell migration. In addition, Kv1.3 and Kv1.5 are remodeled in human lymphomas. Whereas the level of Kv1.3 expression did not correlate with the state of dedifferentiation, Kv1.5 expression was, for the most part, correlated inversely with clinical aggressiveness. In addition, control LNs expressed heterogeneous levels of Kv1.3, whereas Kv1.5 expression was persistently low. Our data therefore identify Kv1.5 as a new target for immunomodulation and provide new insights into the use of Kv1.3 and Kv1.5 as potential targets during tumorigenesis.

MATERIALS AND METHODS

Cell culture, sample preparation, and proliferation assays

Raw 264.7 macrophages, Raji, and Ramos B cells and Jurkat T-lymphocytes were cultured in RPMI culture media, containing 10% FBS and supplemented with 10 U/ml penicillin and streptomycin and 2 mM L-glutamine (complete media). For immunocitochemistry, cells were washed with PBS, fixed in neutral formalin for 5 h, and wrapped in thin cellulose sheets embedded in paraffin for immunohistochemistry (see below).

Enriched murine B- and T-lymphocyte (thymocytes) preparations were obtained from spleen and thymus, respectively. All animal procedures were approved by the Ethics Committee of the University of Barcelona. Mice were killed, and organs were processed under sterile conditions. Spleen and thymus were minced and cell suspension cultured in RPMI as above in the presence of 50 μM 2-ME (Sigma, St. Louis, MO, USA) and 20 μg/μl LPS (Sigma) for B-lymphocyte activation and 0.03 U/μl IL-2 (Gibco, Life Technologies, Carlsbad, CA, USA) and 10 μl/ml anti-CD3/CD28 (Dynabeads mouse T-activator; Invitrogen, Life Technologies, Carlsbad, CA, USA) for T cell activation, respectively.

Proliferation was investigated by analyzing the cell number and viability. Lymphocytes (2×10^5) were cultured in the RPMI culture media in the absence of FBS and supplemented with 0.02% BSA, antibiotics (10 U/ml penicillin and streptomycin), and 2 mM L-glutamine for 24–36 h. Next, starved cells were incubated with complete media and harvested at different times in the presence or absence of MgTx (Alomone Labs, Jerusalem, Israel), Psora-4 (Sigma), and 4-AP (Sigma). A Countess automated cell counter (Invitrogen, Life Technologies) was used for cell counting; cell size and viability were analyzed by means of trypan blue exclusion. Viabilities >85% were used in all groups. In all cases, the number of cells in the presence of FBS was subtracted from the proliferation in its absence. This number is considered 100% of proliferation. In addition, the AlamarBlue dye assay (Life Technologies) was used following the manufacturer's instructions. The AlamarBlue cell viability assay reagent incorporates a redox-sensitive growth indicator to quantitatively measure cell viability and proliferation using fluorescent or colorimetric detection strategies. Results obtained were similar in all cases, and AlamarBlue results from Ramos B cell proliferation (see Fig. 3E) are included for comparison.

RNA isolation, RT-PCR analysis, and real-time PCR

Total RNA from culture cells and mouse tissues was isolated using the NucleoSpin RNA II (Machery-Nagel, Germany). RNA was treated with DNase I, and PCR controls were performed in the absence of RT. cDNA synthesis was performed using transcriptor RT (Roche Applied Science, Indianapolis, IN, USA) with a random hexanucleotide and oligo dT, according to the manufacturer's instructions. Once cDNA was synthesized, the conditions were set for further PCR by using the Phire Hot Start II DNA polymerase (Finnzymes, Thermo Scientific, Finland), following the manufacturer's instructions for 30 cycles (see Supplemental Table 1 for primer information). The final RT-PCR reactions (20 μl) from were electrophoresed in a 1% agarose TBE gel (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0).

Real-time PCR was performed using a 7500 Real-Time System machine with TaqMan Universal Maxter Mix II, no uracil *N*-glycosylase, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Validated human and mouse Kv1.3, Kv1.5, and CXCR5 TaqMan probes (Applied Biosystems) were used on cDNA retrotranscribed from 0.5 μg RNA, as described above. Human RNA was obtained from Stratagene (La Jolla, CA, USA).

Electrophysiological recordings

Currents were recorded using the patch-clamp technique in the whole-cell configuration with a HEKA EPC 10 USB amplifier (HEKA Elektronik, Germany). PatchMaster software (HEKA Elektronik) was used at a stimulation frequency of 50 kHz and filtered at 10 kHz. Capacitance and series resistance compensation were optimized, and usually, 80% compensation of the effective access resistance was obtained. Micropipettes of 3–4 MΩ were fabricated from borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA) using a P-97 puller (Sutter Instrument, Novato, CA, USA). For Raji B cells, electrodes were filled with an intracellular solution containing (in mM): 140 KF, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and 10 Hepes, adjusted to pH 7.20, with KOH (295 mOsm/Kg). The extracellular solution contained (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, and 10 Hepes, adjusted to pH 7.35, with NaOH (305 mOsm/Kg). For Jurkat T cells, the intracellular pipette solution was (in mM): 137 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.2 $CaCl_2$, 10 glucose, 10 Hepes, adjusted to pH 7.4, with NaOH (305 mOsm/Kg), and the intracellular solution was (in mM): 30 KCl, 110 K-aspartate, 2 ATP-K_2, 2 $MgCl_2$, 0.05 EGTA, 10 Hepes, adjusted to pH 7.2, with KOH (295 mOsm/Kg). Raji B cells and Jurkat T cells were clamped at −60 mV, and a 250-ms pulse potential of +70 mV was applied. pA was normalized by pF and expressed as the mean ± SEM. Data analysis was performed using FitMaster (HEKA) and SigmaPlot 10.0 (Systat Software, Chicago, IL, USA). To characterize the K^+ currents pharmacologically, 50 pM and 10

nM MgTx (Alomone Labs), 10 nM and 100 mM Psora-4 (Sigma), and 250 μM and 4 mM 4-AP (Sigma) were added to the external solution. Toxin was reconstituted at 10 μM in Tris buffer (0.1% BSA, 100 mM NaCl, 10 mM Tris, pH 7.5). The inhibition by MgTx, Psora-4, and 4-AP was defined by the remaining current fraction in the presence and absence of the blocker. All recordings were performed at room temperature (21–23°C).

Lentivirus infection

To knock down Kv1.5 in the Ramos-B cell line, Kv1.5 shRNA (human) LTV particles were used (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells (8×10^5) were cultured in 25 cm^2 culture flasks and infected with 50,000 infectious units in the presence of Polybrene (2 μg/ml; Sigma). Twenty-four hours after infection, this medium was replaced by regular medium, supplemented with 10 μg/ml Puromycin (Sigma) for clone selection. Selected clones were maintained with 2 μg/ml Puromycin. Specific silenced expression of Kv1.5, but not Kv1.3, was confirmed by Western blot and PCR. Control shRNA LTV particles containing a Sc sequence were also used.

Cell-cycle analysis and cell size

Cell-cycle analysis was performed with PI [19]. Cells, under different conditions (see above), were collected in 5 ml PBS and spun for 1 min at 200 *g*. Cells were resuspended in 0.5 ml PBS and fixed in ice-cold 70% ethanol for at least 2 h. Fixed cells were next centrifuged at 200 *g* for 5 min, and the pellet was resuspended in 1 ml freshly prepared PI staining solution (20 μg/ml PI/0.1% Triton/0.2 mg DNase-free RNase A). Samples were kept for 30 min at room temperature. Flow cytometric measurements were carried out using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Cell size was measured by the laser light scattered at narrow angles to the axis of the laser beam (forward scatter), which is proportional to the size of the cell, and by a Countess automated cell counter (Invitrogen, Life Technologies).

Transwell migration assays

We performed migration assay by using Boyden chambers. Millicell hanging cell-culture inserts (3 μm pore size, 1.1 cm^2/well; Millipore, Billerica, MA, USA) were used, according to the standard procedures. The cells were seeded on the top chamber at 2×10^7/ml with culture medium. The lower chambers were filled with 500 μl medium, supplemented with FBS and 1 μg/ml CXCL13 (PeproTech, Rocky Hill, NJ, USA). After 24 h, migrated cells were found on the bottom side of the transwell and processed for counting by a Countess automated cell counter (Invitrogen, Life Technologies).

Patients, tissue characteristics, and sample processing

Malignant lymphomas from 29 different patients were obtained from the Department of Pathology of Vall d'Hebron University Hospital (Barcelona, Spain) from 2003 to 2008. For most lymphomas, four independent, individual samples with different histological grades of malignancy were obtained. Patient ages ranged from 15 to 80 years, with a mean age of 55 years. Histologic type and histological grade of malignancy were recorded in a database (Supplemental Table 2). Additionally, four different control LNs of nontumoral samples obtained from lymphadenectomy patients were added to the series as control cases. LN and lymphoma samples were fixed in neutral formalin and embedded in paraffin for immunohistochemistry. In all cases, diagnosis was performed by light microscopy examination, according to current WHO diagnostic criteria, using conventional H&E staining and an ancillary immunohistochemical staining (Supplemental Table 2). Two independent samples for each case were analyzed, and representative results are shown at 400× magnification. This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Ethics Committee of the participating hospital. Informed consent was obtained from each patient or his or her relatives, after full explanation of the procedures.

Antibodies and immunohistochemistry

Immunohistochemical staining using the avidin-biotin-peroxidase technique was performed for each antibody. Samples were deparaffined in xylene and rehydrated in graded alcohol. For antigen retrieval, slices were heated in a pressure cooker in 10 mM citric acid monohydrate, pH 6.0, for 5 min (Kv1.5) or in a 98°C water bath in 10 mM citric acid monohydrate, pH 9.0, for 40 min (Kv1.3). Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxidase-blocking solution for 10 min. Samples were immunoblotted with anti-Kv1.3 (1:70) and anti-Kv1.5 (1:100) polyclonal antibodies (Alomone Labs) for 1 h, followed by incubation with a HRP-labeled polymer anti-rabbit (DakoCytomation, Denmark) for 30 min. Immunohistochemical analysis was performed with the EnVision system (DakoCytomation). Samples were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were performed in all cases by omitting the primary antibody and with the presence of antigen peptides, as described previously [20, 23]. All primary antibodies were also tested by Western blot.

All cases were evaluated by two pathologists semiquantitatively. Whenever a major discrepancy was observed between pathologists, the cases were viewed simultaneously by both pathologists using a multihead microscope, and a consensus was reached.

Protein extraction and Western blot analysis

Biopsies were frozen immediately and kept at −80°C until use. Human samples and culture cells were homogenized with ice-cold lysis solution (1% Triton X-100, 50 mmol/l Tris HCl, pH 7.5, 150 mmol/l NaCl, 1 mM EDTA), supplemented with 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF as protease inhibitors. Homogenates were centrifuged at 5000 *g* for 10 min, and the supernatants were stored at −80°C. Protein content was determined by a Bio-Rad (Hercules, CA, USA) protein assay.

Protein samples (50 μg) were boiled in Laemmli SDS loading buffer and separated on 10% SDS-PAGE. They were transferred to Immobilon-P membranes (Millipore) and blocked in 5% dry milk, supplemented with 0.05% Tween 20 in PBS, before immunoreaction. Filters were immunoblotted with antibodies against Kv1.3 (1:200) and Kv1.5 (1:500). Anti-β-actin mAb (1:50,000; Sigma) was used as a control. Secondary antibodies were from Bio-Rad (anti-rabbit, 1:3000; anti-mouse, 1:10,000). Densitometric analysis of the filters was performed by Phoretix software (Nonlinear Dynamics, Newcastle upon Tyne, UK), and values were normalized to the relative β-actin expression.

Immunohistochemistry evaluation

All cases were evaluated by pathologists, taking into account the percentage of positive TCs and intensity of the staining. Kv1.3 and Kv1.5 were evaluated by calculating a Hscore: Hscore = (1×% weak-staining cells) + (2×% moderate-staining cells) + (3×% strong-staining cells), with results ranging from 0 to 300, as described previously [24]. Weak-staining cells were marked as +; moderate, ++; and strong, +++. High Hscore values represent major prevalence of staining. In randomly chosen cases, the immunostaining was performed in complete tissue sections to evaluate the consistency of the results.

Statistical analysis

Where appropriate, values were expressed as the mean ± SEM. Significance was tested by one-way ANOVA with a Tukey post-test and Student's *t*-test (GraphPad Prism, GraphPad Software, San Diego, CA, USA). Furthermore, the Pearson's correlation coefficient was calculated where indicated. A value of $P < 0.05$ was considered significant.

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RESULTS

Kv1.5 is expressed in human B-lymphocytes and contributes to B cell proliferation

Human lymphocytes express Kv channels that participate in cell proliferation and activation [1, 13]. Therefore, we characterized the voltage-dependent K^+ currents in Jurkat T cells (**Fig. 1**). Depolarizing steps from −60 mV to +70 mV elicited outward K^+ currents that were inhibited by 4-AP in a dose-dependent manner (Fig. 1A, E, and F). Kv currents in Raji-B cells exhibited a similar behavior (Fig. 1B, E, and F). As 4-AP is a general K^+ channel blocker, we next used more selective compounds to further characterize K^+ currents. Thus, MgTx for Kv1.3 and Psora-4, highly specific for Kv1.3 and Kv1.5 [25], were used (Fig. 1C, D, H, and I). Whereas MgTx potently blocked K^+ currents in T cells, K^+ currents were more MgTx-resistant in Raji lymphocytes (Fig. 1I). However, Psora-4 was highly effective in B cells (Fig. 1D, H, and I). Our results suggested that human B-lymphocytes expressed a K^+ current that was resistant to MgTx but was inhibited efficiently by Psora-4.

To characterize further the Kv responsible for the K^+ currents in human B-lymphocytes, we performed RT-PCR assays. **Figure 2A** shows the gene expression of some Kv channels that have been detected in leukocytes [5, 26–28]. Whereas Kv1.1, Kv1.2, Kv1.3, Kv1.6, and Kv3.1 were present in Jurkat T-lymphocytes, Raji B cells only expressed Kv1.3, Kv1.5, and Kv3.1 (Fig. 2A). The absence of Kv1.5 in T cells but not in B cells is noteworthy.

Abundant data confirm the predominant expression of Kv1.3 in immune system cells, not only in lymphoblastic leukemia cell lines but also in primary cultures [1, 21]. In addition to Kv1.3, cells from the myeloid origin express Kv1.5 [4–7, 11]. The expression of Kv1.5 is, for the most part, related to a negative effect on the Kv1.3-dependent control of the immune response [6, 8, 9, 29]. Although the presence of both subunits is not under debate in macrophages, we confirmed the protein expression of Kv1.5 in Raji B-lymphocytes but not in Jurkat T cells (Fig. 2B). Similar results were obtained with the parental human B cell line Ramos (not shown). This pattern was confirmed further by immunocytochemistry (Fig. 2C).

The K^+ channel, involved in the proliferation of B-lymphocytes, remained elusive [13–15]. Whereas Kv1.3 controls the proliferation of T cells and macrophages, Kv1.5 regulates the cell cycle in myocytes [1, 4, 19]. Therefore, we analyzed whether Kv1.5 participates in B cell proliferation. 4-AP (4 mM) and 100 nM MgTx inhibited T cell proliferation (**Fig. 3A**). In addition, 1 μM Psora-4 inhibited proliferation of Jurkat cells by 30%. Interestingly, 4-AP and Psora-4, but not MgTx, blocked the proliferation of human Raji B cells (Fig. 3B). Whereas higher doses of MgTx did not impair B cell proliferation (Fig. 3C), 4-AP inhibited Raji cell proliferation in a dose-dependent manner (Fig. 3D). The putative role of Kv3.1 in B cell proliferation seemed minor, as doses of 4-AP that were tenfold higher than the IC_{50} [30] triggered no effect. Similar results were obtained in Ramos B cells (Fig. 3E). In addition, Fig. 3F demonstrates that whereas 4-AP and Psora-4 arrested Ramos B cells in the G1-phase of the cell cycle, the presence of 100 nM MgTx did not alter cell-cycle progression.

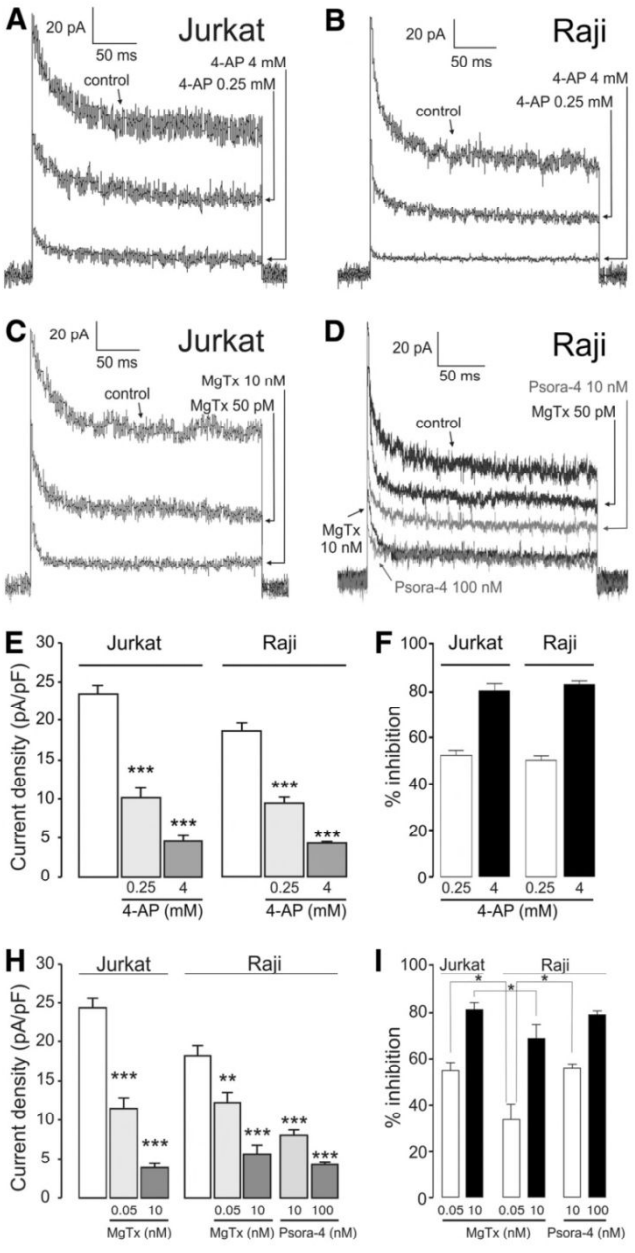

Figure 1. Pharmacological characterization of voltage-dependent K^+ currents in Jurkat T- and Raji B-lymphocytes. Cells were held at −60 mV, and a 250-ms pulse potential of +70 mV was applied. (A and B) Representative traces of delayed rectifier K^+ currents in the presence or absence of 0.25 and 4 mM 4-AP in Jurkat (A) and Raji (B) cells. (C) Representative traces of delayed rectifier K^+ currents in Jurkat cells in the presence or the absence of 50 pM and 10 nM MgTx. (D) Representative traces in Raji cells in the presence or the absence of 50 pM and 10 nM MgTx (black line) and 10 nM and 100 nM Psora-4 (gray line). (E and H) pA was normalized by pF and expressed as the mean ± SEM in the presence or the absence of 4-AP (E) and MgTx or Psora-4 (H). (F and I) Percentage of inhibition at the peak current density (+70 mV) in the presence of 4-AP (F) and MgTx or Psora-4 (I). (E and H) $^{**}P < 0.01$; $^{***}P < 0.001$ versus no additions. (I) $^{*}P < 0.05$ Raji versus Jurkat or Psora-4 versus MgTx in Raji; $n = 6–8$ cells/group (Student's *t*-test).

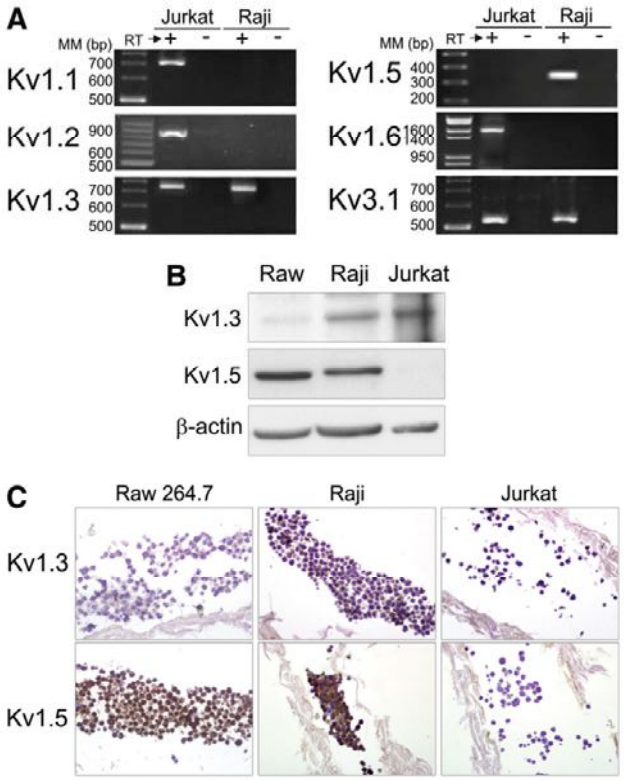

Figure 2. Kv expression in Jurkat and Raji lymphocytes. (A) mRNA expression of Kv1.1, Kv1.2, Kv1.3, Kv1.5, Kv1.6, and Kv3.1. RT-PCR reactions were performed in the presence (+RT) or absence (−RT) of retrotransciptase. (B) Kv1.3 and Kv1.5 protein expression in Raw 264.7 macrophages, Jurkat T cells, and Raji B cells. β-Actin was used as a transfer and loading control. (C) Immunocytochemistry of Kv1.3 and Kv1.5 in Raw 264.7 macrophages, Raji B-lymphocytes, and Jurkat T cells; upper, Kv1.3; lower, Kv1.5. Kv1.3 and Kv1.5 were analyzed in two independent slices. Representative images are shown.

To characterize further the role of Kv1.5 in human B cell proliferation, we generated a Kv1.5-deficient Ramos cell line (LTVKv1.5). Infection with Kv1.5 shRNA (human) LTV particles depleted Kv1.5 expression by 70% (**Fig. 4A**). HEK-293 and Raw 264.7 macrophages were included as negative and positive controls, respectively. LTV particles, containing a scrambled shRNA sequence, were also used to generate a control cell line (Sc). The depletion of Kv1.5 caused a 40% inhibition of FBS-dependent proliferation of Ramos B cells (Fig. 4B). This effect was more evident earlier in LTVKv1.5 cells when FBS was reintroduced (Fig. 4C), yielding a slower proliferation rate and arresting LTVKv1.5 cells in the G1-phase of the cell cycle (Fig. 4D).

Kv1.3 is involved in macrophage migration [8]; as cell migration is one of the key features of neoplastic cell growth and invasion, we studied the migration capacity of LTVKv1.5 B cells (Fig. 4E). Depletion of Kv1.5 triggered a threefold increase in the migration of Ramos B cells (Fig. 4E) and increased CXCR5 mRNA abundance, a receptor implicated in B cell migration (not shown). Furthermore, this was not a consequence of the cell size (Fig. 4F). These data indicated that Kv1.5 plays a physiological role during proliferation and migration of B-lymphocytes.

Expression of Kv1.3 and Kv1.5 in the immune system

The role of Kv1.5 in human B-lymphocyte physiology had not been reported previously. Therefore, we analyzed the gene expression of Kv1.5, as well as Kv1.3, in a number of human and mouse tissues and primary cells (**Fig. 5**). Our results demonstrated that Kv1.5 was widely distributed throughout the immune system. Thus, in addition to Raji B cells, Kv1.5, along with Kv1.3, is expressed in the LN, lymphoma, thymus, and spleen. Noteworthy is the change in the expression pattern between the human LN and a malignant non-Hodgkin lymphoma specimen. Kv1.5 was also differentially expressed in mouse lymphoid tissues and native lymphocytes. Interestingly, whereas the expression of Kv1.3 and Kv1.5 increased in primary B cells during LPS and β-ME expansion, the expression of Kv1.5 is notable after 2 days of IL-2 and anti-CD3/CD28 differentiation of thymocytes in culture. Therefore, the expression of Kv1.5 in the immune system should be considered more ubiquitous than previously thought.

Phenotypical characterization of human LNs and lymphoma samples

The role of Kv1.3 and Kv1.5 during carcinogenesis is controversial [21, 22, 31]. Furthermore, we found a remodeling of both channels in a non-Hodgkin lymphoma specimen of unknown diagnosis (Fig. 5). Therefore, we wanted to analyze, for the first time, the expression of both proteins during neoplastic cell growth in human non-Hodgkin lymphomas.

We first undertook a preliminary phenotypical characterization of our control LNs to map the regional cell differences in our human specimens. As expected, LNs were positive for CD3 (T cells), CD20 (B cells), CD68, MPO (myeloid), CNA42 (follicular dendritic), and CD138 (mature plasmatic cells; Supplemental Fig. 1A–F). Thus, although there were specific differences in the regional localization of myeloid and lymphoid cells, Kv1.3 and Kv1.5 thoroughly stained the samples with minor differences (Supplemental Fig 1G and H).

To classify human biopsies, in addition to the WHO criteria, we analyzed the expression of several markers prior to an extensive Kv1.3 and Kv1.5 study in a number of human lymphomas. Several clusters of differentiation proteins [CD3, CD5, CD10, CD20, CD30, CD45 (CD45RO and CD45RA), CD56, CD79, and CD138], as well as a plethora of selected proteins (bcl2, bcl6, Ki67, p53, latent membrane protein 1, cyclin D1, ALK-1, MUM-1 and EMA) were used. Supplemental Table 2 shows the major histochemical characteristics of the lymphomas.

DLBCL were mostly identified by the presence of CD20, CD10, bcl6, and MUM-1 and the absence of CD3, CD5, and bcl2. The presence of CD10, CD20, and bcl6, in combination with bcl2, characterized F. MCL were identified by the expression of CD5, CD20, and cyclin D1. In addition to CD5 and CD30, the presence of EMA and ALK-1 pointed to

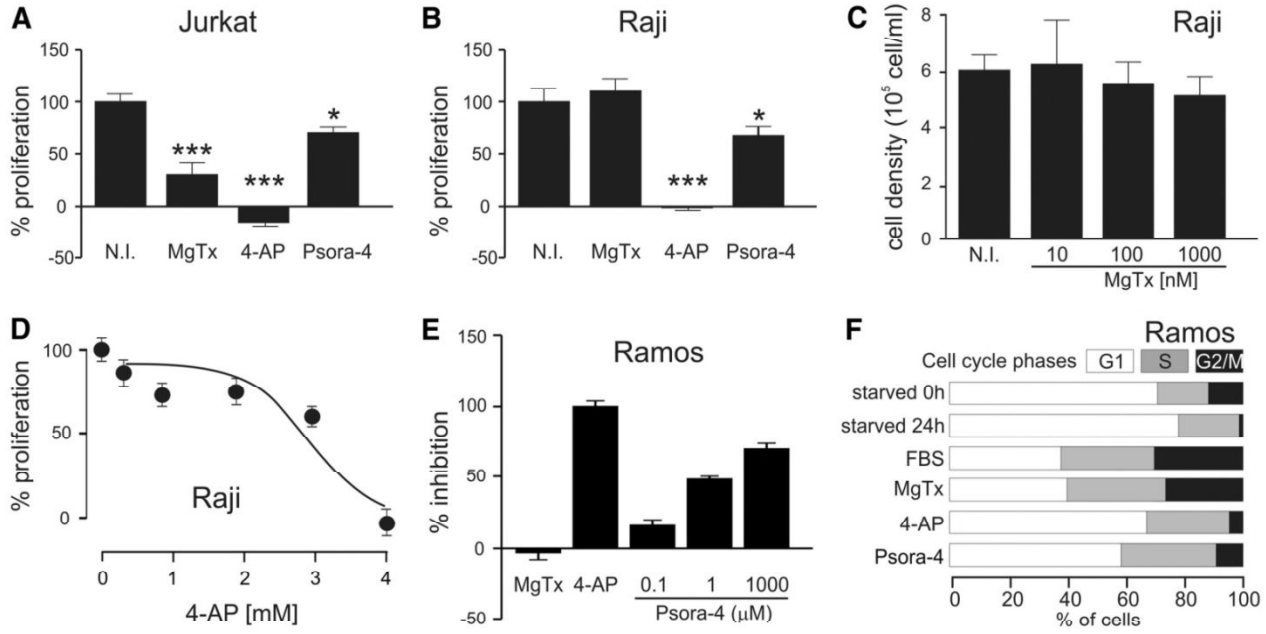

Figure 3. Kv1.5 participates in the proliferation of human B-lymphocytes. Resting cells cultured for 24–36 h without serum (supplemented with 0.02% BSA) were incubated for the next 24 h in the presence of serum without [no inhibitors (N.I.)] or with 100 nM MgTx, 4 mM 4-AP, or 1 μM Psora-4. (A) Percentage of proliferation of Jurkat T cells. (B) Percentage of proliferation of Raji B cells. $^*P < 0.05$; $^{***}P < 0.001$ versus no inhibitors (FBS alone); $n = 4–6$ independent experiments (Student's t-test). (C) Dose effects of MgTx on Raji cell density. (D) Dose effects of 4-AP on Raji cell proliferation. (E) Effects of several K^+ channel blockers on the human Ramos B cell proliferation analyzed by the AlamarBlue dye assay. The AlamarBlue reagent is a nontoxic, water-soluble resazurin dye that yields a fluorescent signal and a colorimetric change when incubated with metabolically active cells. The reagent is designed for cell viability and proliferation studies using mammalian cell lines. Cells were treated as in B. Percentage of inhibition was calculated in the presence of 100 nM MgTx, 4 mM 4-AP, and several doses of Psora-4. Values are the mean ± SEM of four different assays, each performed in triplicate. (F) Cell-cycle analysis of Ramos B cells in the presence or absence of 100 nM MgTx, 4 mM 4-AP, and 1 μM Psora-4. Cell-cycle distribution (%) after 24 h in the absence (starved 0 h and 24 h) or the presence of FBS. Starved 0 h represents resting cells cultured without FBS for 36 h before addition of FBS (time 0 h of the experiment). Starved 24 h represents resting cells cultured in the absence of FBS during 36 h that were kept without FBS for another 24 h throughout the experiment (60 h total in the absence of FBS). G1-phase, white; S-phase, gray; G2/M-phase of the cell cycle, black.

ALCL. Finally, whereas T expressed CD3, CD30, CD56, and CD45RO, they were negative for the identification of CD20 and EMA. Other markers revealed only a minor presence of other inflammatory and plasma cells (Supplemental Table 2).

To establish a phenotypic correlation of clinical aggressiveness, a Hscore, based on the expression of Ki67 and p53, was performed (Supplemental Fig. 2). Hscore has been validated as a semiquantitative analysis for the evaluation of protein expression by immunohistochemistry [24]. Supplemental Fig. 2B highlights a significantly close relationship between p53 and Ki67 expression, which ranged from the indolent F to the most aggressive ALCL ($P<0.037$; Pearson's correlation coefficient, $r=0.843$).

Kv1.3 and Kv1.5 abundance in human lymphomas

Data in Fig. 5 revealed that Kv1.3 and Kv1.5 shared expression in human lymphomas and most importantly, mRNAs remodeled during neoplastic growth. Therefore, we performed a screening of human samples analyzed by Western blot (**Fig. 6**). Up to four individual specimens for each lymphoma and control LN were processed. Two different Western blots, by duplicate, were performed in parallel, and several samples were used as internal controls in both filters. Thus, four N (N^{1-4}), four DLBCL (B^{1-4}), one ALCL, one MCL, one F (F^4), and one T (T^2) were in Filter 1 (Fig. 6A, left), and one N (N^1), one MCL, three F (F^{1-3}), and three T (T^{1-3}) were analyzed in membrane two (Fig. 6A, right). Thus, some samples were shared by both filters: MCL, N^1, F^4, and T^2. Therefore, results in Fig. 6B and C represent the relative abundance of Kv1.3 and Kv1.5, taking into account, first, the abundance of β-actin and second, the relative expression of both channels in the MCL sample that showed the lower expression for Kv1.3 and Kv1.5 (see below).

The abundance of Kv1.3 in control LNs was heterogeneous, which could indicate different levels of reactivity. Kv1.3 staining was more uniform in DLBCL and F. Kv1.3 abundance was also uniform but lower in T and in ALCL and MCL. The low-

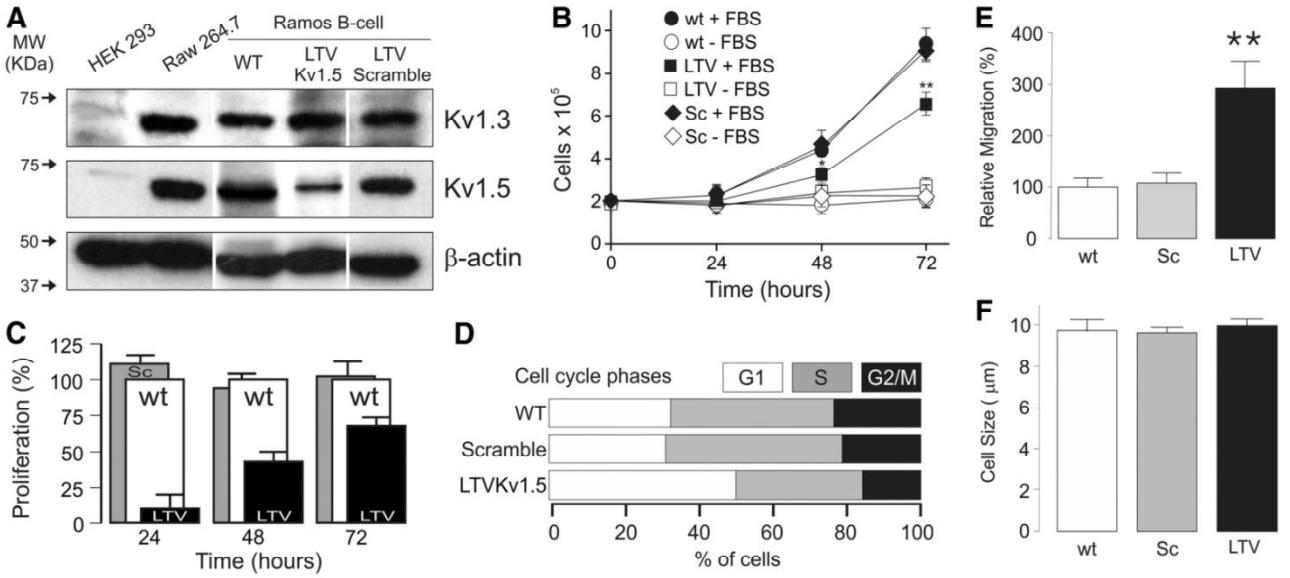

Figure 4. Kv1.5 gene silencing alters B-lymphocyte proliferation and migration. LTV shRNA (human) to Kv1.5 was used to silence the Kv1.5 gene in human Ramos B cells. (A) Representative Western blots from multiple experiments. HEK-293 cells and Raw 264.7 macrophages were used as negative and positive controls, respectively, for Kv1.3 and Kv1.5 expression. Whereas Ramos WT cells and LTV Sc expressed similar levels of Kv1.3 and Kv1.5, LTVKv1.5 (LTV) cells showed lower Kv1.5 abundance. (B) Proliferation of Ramos WT, Sc, and LTVKv1.5 cells in the presence (+FBS, closed symbols) or the absence (−FBS, open symbols) of FBS. Ramos WT, circles; Sc cells, diamonds; LTVKv1.5 cells, squares. $*P < 0.05$; $**P < 0.01$ versus LTVKv1.5 + FBS. (C) Relative percentage of proliferation (%). The FBS-dependent proliferation of Ramos WT cells was kept as the reference and compared with the relative percent of proliferating Sc and LTVKv1.5 cells at 24, 48, and 72 h. (D) Cell-cycle analysis of Ramos B cells (WT), LTV Sc, and LTVKv1.5 cells. Cell-cycle distribution (%) after 24 h in the presence of FBS. G1-phase, white; S-phase, gray; G2/M-phase of the cell cycle, black. (E) Relative migration (%) of Ramos WT, LTV Sc, and LTVKv1.5 cells. $**P < 0.01$ versus Ramos WT (Student's *t*-test). (F) Cell size of Ramos WT, LTV Sc, and LTVKv1.5 cells. Ramos WT, white bars; LTV Sc, gray bars; LTVKv1.5, black bars. All values are the mean ± SEM of four to six different assays performed in triplicate.

est expression in both filters corresponded to the MCL specimen; therefore, this sample was taken as a reference. Figure 6B shows the individual quantification of Kv1.3 results shown in Fig. 6A. Although variability was observed among the samples, overall results showed a defined Kv1.3 expression pattern (MCL<ALCL<T<F<N=DLBCL).

Kv1.5 also showed differential expression. Whereas N, DLBCL, and MCL shared a low expression, ALCL, F, and T expressed higher levels of Kv1.5 (Fig. 6A and C). Unlike Kv1.3, the abundance of Kv1.5 was more homogeneous among different N and DLBCL samples. The differential relative abundance of Kv1.5 ranged MCL = N < DLBCL < T = ALCL < F.

To analyze whether Kv1.3 and Kv1.5 levels were somehow related with the nature of the tumor, we plotted the Kv1.3 and Kv1.5 relative expression versus the clinical aggressiveness of the lymphoma following the WHO lymphoma classification (Fig. 6D), which was supported further by the expression of p53 and Ki67 (Supplemental Fig. 2). Although the Kv1.3 pattern was quite heterogeneous, Kv1.5 expression (F>T=ALCL>DLBCL) fitted inversely to the aggressiveness ($P<0.018$, one-way ANOVA; $P<0.05$ F vs. T and DLBCL by Tukey post-test). Interestingly, although Kv1.3 was heterogeneously high in control LN, Kv1.5 remained homogeneously low.

Immunohistochemistry and Hscore evaluation of Kv1.3 and Kv1.5 in lymphomas

Data in Fig. 6 suggested no correlation between the Kv1.3 expression and the tumor aggressiveness. In this context, Supplemental Fig. 3A–F shows representative images of the expression of Kv1.3 in human LN biopsies and lymphoma specimens. Control LNs heterogeneously stained for Kv1.3. Following the phenotypical characterization in Supplemental Fig. 1, although T were weakly labeled (+), plasmatic B and/or myeloid cells (B) were strongly stained (+++). A similar phenotype was found within proliferating cells in the GC.

Unlike N specimens, human lymphomas were homogeneously stained with Kv1.3. Thus, DLBCL (+ in 98% of TC), F (++ in 95% TC), MCL (++ in 85% TC), T (++ in 95% TC), and ALCL (++ in 95% TC) showed Kv1.3 staining. Concomitantly, the Kv1.3 Hscore evaluation showed no major differences among lymphomas (Supplemental Fig. 3G). Furthermore, similar to that observed in Fig. 6D, Kv1.3 expression showed no correlation with tumor aggressiveness (Supplemental Fig. 3H).

In addition to Kv1.3, macrophages and B-lymphocytes express Kv1.5 (see Fig. 2), which plays a role during activation and proliferation. **Figure 7** shows representative pictures of

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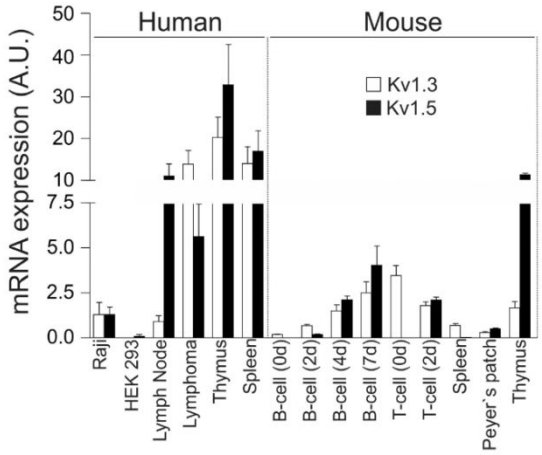

Figure 5. Kv1.3 and Kv1.5 mRNA expression in various human and mouse tissues and cells. Real-time PCR was performed, and relative abundance in arbitrary units (A.U.) calculated, with the expression of Kv1.3 and Kv1.5 in human Raji B cells as the reference. Abundance of 18S was considered an internal control as described previously [9]. Commercial human RNA was from Stratagene. Enriched population of mouse B cells from spleen and T cells (thymocytes) from thymus was activated in culture during several days (0–7 days and 0–2 days, respectively), as described in Materials and Methods. Kv1.3, white bars; Kv1.5, black bars. Values are the mean ± SEM of three different assays, each performed in triplicate.

Kv1.5 expression in control LN and lymphoma specimens. Kv1.5 staining was moderate and shows compartmentalization between the GC and the rest of the tissue (Fig. 7A). TB in DLBCL were homogenously (90% of TC) and moderately stained (++) by Kv1.5 (Fig. 7B). However, the Kv1.5 label in the rest of noncancerous lymphocytes was heterogeneous, and some T cells were negative (T). In indolent F, which contain highly differentiated B cells, the Kv1.5 signal was strong (+++) and homogeneous (90% of TC; Fig. 7C).

MCL was heterogeneously stained by Kv1.5 (Fig. 7D), and only ~50% of lymphomatous B cells were stained (++). Our T specimens were also heterogeneously stained by Kv1.5 (Fig. 7E). The Kv1.5 signal was moderate (++) in 50% of TC. In addition, in many Kv1.5-positive TC, mitotic events were observed (M). Finally, the ALCL biopsy revealed a heavy (+++) expression of Kv1.5 in the majority of big and unshaped TC (95%). In addition, many of the nontumoral lymphocytes, mostly T cells, were negative (Fig. 7F). Figure 7G shows a graphical representation of Hscore for Kv1.5 in all lymphoma samples. Unlike Kv1.3, Kv1.5 expression followed a statistically inverse correlation ($P<0.040$; Pearson's correlation coefficient, $r=0.895$) with the clinical aggressiveness of the tumor (Fig. 7H).

DISCUSSION

Many Kv contribute to the cell-cycle progression in mammalian cells [22, 31–35]. There are several events that Kv may control during cell growth, such as membrane potential, cell volume and Ca^{++} signaling [31, 32, 34, 36]. Although highly proliferative cells are depolarized, during the G1/S-phase transition of the cell cycle, a transient hyperpolarization is needed. In addition to cell proliferation, Kv channels contribute to cell activation, apoptosis, and differentiation, especially in cells from the immune system [1, 35]. Leukocytes express a limited Kv repertoire, and Kv1.3 and Kv1.5 play major roles [1–4]. As Kv1.3 and Kv1.5 have been implicated in cell proliferation and activation in the immune system, these channels are good candidates for being involved in neoplastic lymphomatous processes.

In the present study, we demonstrate that Kv1.5 is expressed in human B-lymphocytes and participates in cell proliferation and migration. Although Kv1.3 is expressed in B cells, preliminary results were controversial, as 4-AP and quinine, but not CTX, inhibit B-lymphocyte proliferation [13, 14]. Whereas 4-AP is a general Kv blocker, quinidine, a quinine derivative, is highly efficient in inhibiting Kv1.5 [19, 37]. These results argue against a role of Kv1.3 in B cell proliferation and activation [13, 14]. The limited effect of CTX on proliferation reflects a limited ability to depolarize human peripheral blood lymphocytes [15, 38]. However, high extracellular $[K^+]$, which causes depolarization, halts B cell proliferation [15]. In this context, membrane depolarization selectively inhibits Kv1.5 in pituitary cells [16]. Our results highlight this issue and demonstrate that Kv1.5 plays a role in the proliferation of human B-lymphocytes. In this scenario, B-lymphocytes share Kv1.5 expression with macrophages and DCs [5, 7, 29]. Kv1.5 acts as a negative subunit, fine-tuning the immune response in mononuclear phagocytes [9]. However, in B cells, Kv1.5 plays major and definitive roles similar to muscle cells [19]. Although Kv1.5 was necessary for B cell growth, channel silencing improved cell migration. Both of the features are characteristic of tumor aggressiveness. In addition to proliferation, inflammatory cell infiltration is an important characteristic of metastasis and tumor progression. By inhibiting Kv1.3, migration of macrophages is impaired [8]; however, by depleting Kv1.5, B cell migration is improved. It is therefore tempting to speculate that similar to macrophages, the Kv1.3/Kv1.5 ratio may determine the level of cell activation [6]. Similar to the results in macrophages [4, 6], B-lymphocyte proliferation did not increase Kv1.5 expression (not shown).

As Kv1.5 is expressed more ubiquitously in the immune system than previously thought and as B cells and macrophages share Kv1.3 and Kv1.5, both of which are actively involved in proliferation, two important questions arise: (1) are Kv1.3 and Kv1.5 involved in carcinogenesis? and (2) is there any relationship between Kv1.3 and Kv1.5 expression and clinical aggressiveness of human lymphomas?

Kv1.3 and Kv1.5 were differentially expressed in non-Hodgkin human lymphomas. DLBCL, F, MCL, ALCL, and T shared no characteristic pattern. However, considering the prognosis of the tumor, although Kv1.3 showed no apparent connection, Kv1.5 expression was inversely correlated with aggressiveness. Concomitantly, whereas control LNs obtained from patients under metastasis evaluation expressed high levels of Kv1.3, Kv1.5 abundance was low. In this context, the Kv1.3/Kv1.5 ratio may be considered a good indicator of cell activation, and

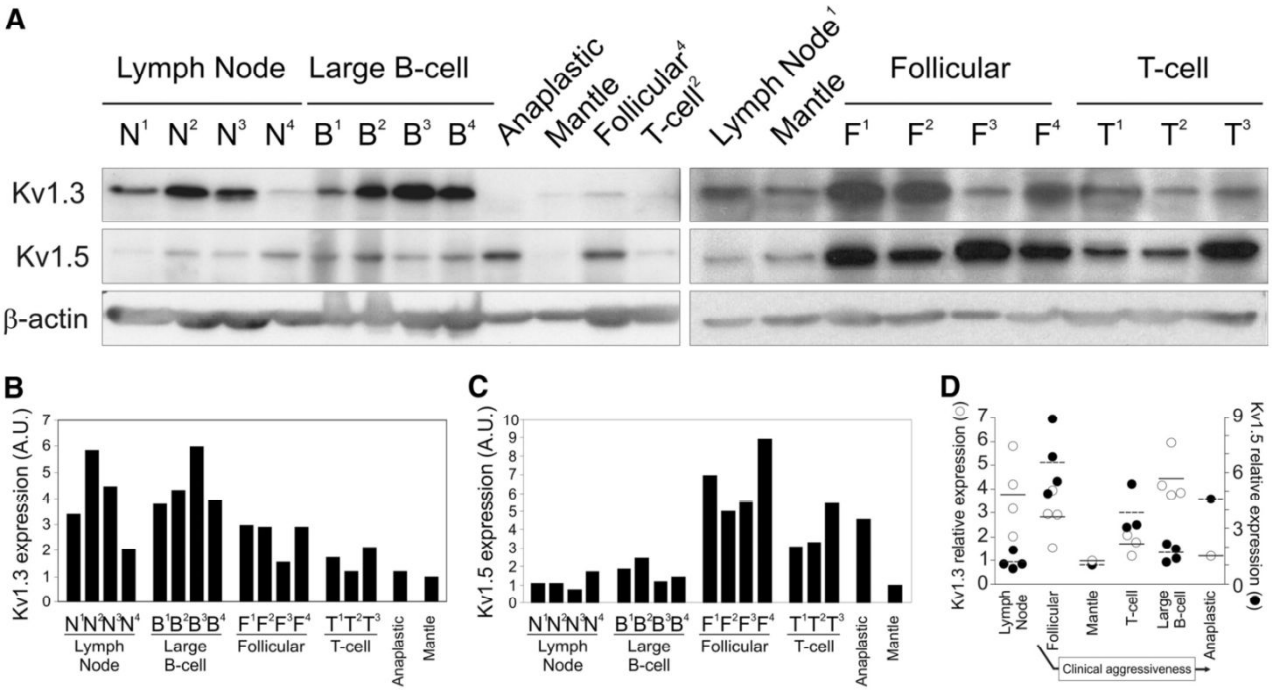

Figure 6. Kv1.3 and Kv1.5 expression in human LN and lymphomas. (A) Representative Western blot analysis of individual samples. Alterations of background levels of all blots were performed consistently in all panels. (Left and right) Filters 1 and 2, respectively. Both filters share ALCL, MCL, F (F^4), T (T^2), and N (N^1) specimens for comparison. (B) Relative Kv1.3 expression of individual samples. (C) Relative Kv1.5 expression of individual samples. Densitometric analysis was performed, taking into account the relative β-actin abundance for each sample and the expression of the channel in the MCL, as described in Materials and Methods. (D) Relative expression of Kv1.3 (open circles) and Kv1.5 (closed circles) in individual samples versus the clinical aggressiveness of the lymphoma. The average is represented by solid and dashed lines for Kv1.3 and Kv1.5, respectively. In all cases, the expression of both proteins in the MCL specimen was taken as reference. One-way ANOVA for all lymphomas indicated an inverse correlation between Kv1.5 abundance and the clinical aggressiveness of the lymphoma ($P<0.018$). A further Tukey post-test analysis also showed significant differences between F and T ($P<0.05$) and DLBCL ($P<0.05$).

high levels of Kv1.5 point to an immunosuppressive state of the cell [6, 8]. However, increased Kv1.3/Kv1.5 ratios indicate cell activation [6, 8]. In addition, whereas the staining for Kv1.5 was homogeneous, the signal for Kv1.3 was highly heterogeneous, demonstrating the different state of activation in the cell lineages within the LN.

As Kv1.3 and Kv1.5 are clearly involved in proliferation, both proteins have been studied in various cancers [20–22]. Surprisingly, despite their contribution to leukocyte physiology, no previous study has addressed human lymphomas. This lack of data has led to a debate in the field and warrants further research. A recent study from our lab, which analyzed a wide variety of human tissues and their neoplastic counterparts, indicated that whereas Kv1.3 shows no apparent relationship with tumorigenicity, most cancers present an aberrant Kv1.5 expression [20]. We have demonstrated that Kv1.3 is remodeled with no correlation with lymphoma aggressiveness. Kv1.3 has been studied in depth in gliomas and in cancers of the pancreas, colon, prostate, and breast [39–44]. Whereas Kv1.3 increases in mammary gland carcinoma and colon cancer, its expression decreases in prostate cancer [39–41]. In addition, Kv1.3 has been documented in several gliomas, with no correlation between channel expression and malignancy [44]. Our results highlight the expression of Kv1.3 in numerous infiltrating inflammatory cells surrounding the tumor nodule as a putative source of variation [20]. Based on these studies, it is not clear whether Kv1.3 is expressed at significant levels in all cancer cells or whether expression is dependent on the stage of the cancer. Although Kv1.3 has been suggested to promote proliferation [45], its function during cell growth is debatable, as it also controls activation and is crucial for the induction of apoptosis in lymphocytes [46–48]. These data suggest multiple roles for Kv1.3 that may depend on the stage of the cancer and the degree of activation of accompanying and resident cells. In this scenario, our results are of interest. Not only lymphoma biopsies but also N samples expressed variable levels of Kv1.3. However, reactive control LNs could mask the results in some specimens.

Unlike Kv1.3, Kv1.5 expression showed an inverse correlation with lymphoma aggressiveness. Whereas high Kv1.3/Kv1.5 ratios facilitate migration, differentiation, and activation of leukocytes, low Kv1.3 activity, caused by Kv1.3 antagonists or immunosuppressive insults, impairs physiological responses [6, 8]. For instance, control N specimens that are reactive and

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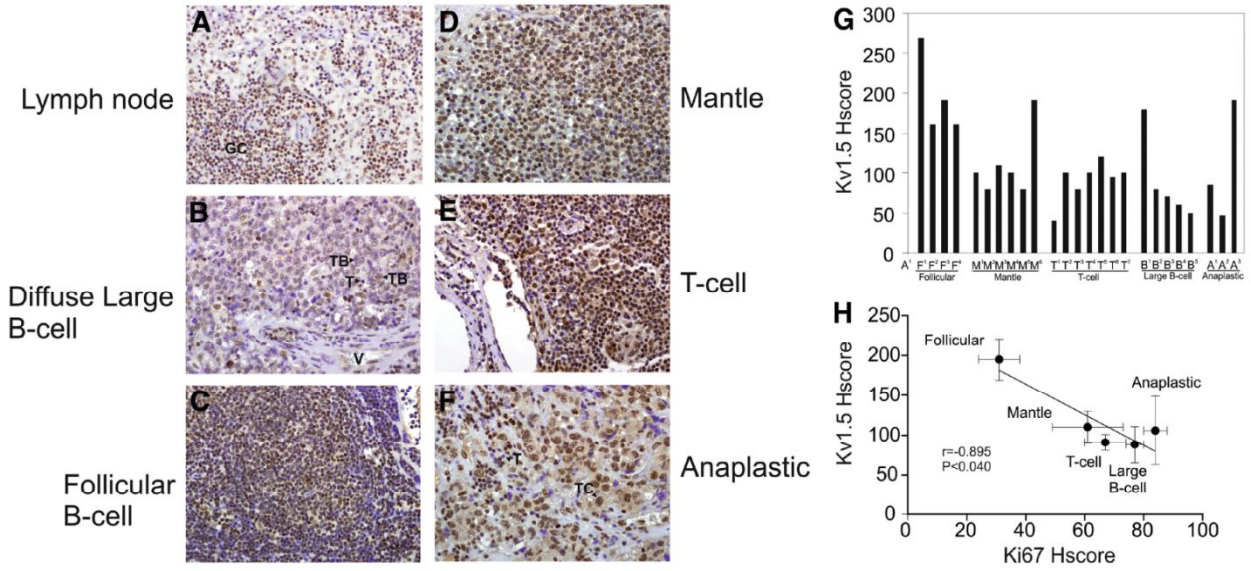

Figure 7. Immunohistochemistry of Kv1.5 in human LN and lymphoma specimens. Kv1.5 was analyzed in two different slices from up to four independent specimens. Representative images are shown. (A) Axillary LN. Sample information: negative for metastasis from a breast cancer patient. (B) DLBCL. Sample information: big cell lymphoma tumor with marked necrosis areas (not in the picture). TB, TBs with large nuclei; T, normal nontumoral accompanying T cells; V, blood vessel. (C) F. Sample information: homogeneous specimen with a well-differentiated follicle. (D) MCL. Sample information: negative for CD10 and CD3. (E) T. Sample information: heterogeneous pleomorphic medium/large T, lymphocyte depletion. (F) ALCL. Sample information: this specimen expressed more T than B cell markers. TC, Non-T, non-B big TCs; T, normal nontumoral accompanying T cells. (G) Hscore of Kv1.5 in individual human lymphomas. Hscore was calculated as described in Materials and Methods. (H) Mean ± SEM of Kv1.5 Hscore plotted against Ki67 Hscore as a marker of the clinical aggressiveness of the lymphoma. A Pearson's correlation coefficient of $r = 0.895$ with a $P < 0.040$ indicates an inverse correlation between Kv1.5 abundance and the aggressiveness of tumor. Values are mean ± SEM of Kv1.5 and Ki67 (see Supplemental Fig. 2) Hscores.

show subsets of activated cells also present low and homogeneous levels of Kv1.5. Although an increase in Kv1.5 may be considered a main feature of the other types of carcinomas, it should be noted that in some cases, this increase is a result of the presence of infiltrating inflammatory cells [20]. Similarly, aggressive lymphomas had a lower and homogeneous abundance of Kv1.5. Preussat and coworkers [44] found a similar inverse correlation between the level of Kv1.5 immunostaining and tumor grade in gliomas. Therefore, the level of Kv1.5 protein in lymphoid neoplasias may be useful in prognosis and outcome prediction.

AUTHORSHIP

A.V-G., J.B., and N.C. performed research on culture cell lines. J.B. and J.H-L. performed research on human lymphomas. J.C., M.C.R-M., and J.C.F. analyzed human lymphomas. J.C.F., S.R.C., and E.C. collected human biopsies and provided reagents. J.M. and C.S. performed research in mouse and human native tissues and cells. A.F. designed and managed the study, participated in data analysis, and wrote the manuscript. All authors contributed to discussion.

ACKNOWLEDGMENTS

The paper was supported by MINECO, Spain (BFU2008-00431, BFU2011-23268, and CSD2008-00005 to A.F. and BFU2010-15674 to C.S.). A.V-G. and J.B. hold fellowships from MINECO. We also thank the editorial assistance of American journal experts.

DISCLOSURES

The authors declare no conflict of interest.

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KEY WORDS:
B cell · cancer · proliferation · migration · potassium channels

3.1.2. Contribution 2.

K_v1.3 and K_v1.5 in cancer models: glioblastoma and pancreatic ductal adenocarcinoma (PDAC).

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SUMMARY

Membrane ion channels participate in the control of cell proliferation and some K_v channels seem to be related with cancer development. A lot of analysis suggests that tumour cells contain an aberrant expression of K_v channels. Because K_v1.3 and K_v1.5 remodelled during tumorigenesis, the objective of this work was to analyse the expression of both channels in human gliomas and pancreatic. The aim of our studies was to analyse the potential role and implication of the voltage dependent potassium channels K_v1.3 or K_v1.5 in human glioblastoma or pancreatic tumours. We wondered whether K_v1.3 or K_v1.5 protein expression correlated with malignancy degree or tumour aggressiveness. Our studies indicated that K_v1.3 and K_v1.5 channels are both present in human glioblastoma samples and that K_v1.5 channels is clearly expressed in PDAC cell lines. However, they did not seem to be playing a pivotal role in proliferation or migration in the pancreatic cancer model. The use of more general pharmacological tools than the specific Psora-4, as 4-AP, opened the possibility that other K_v1 or Shaker channels, rather than the K_v1.3 and K_v1.5, could be involved in those processes. This anomalous expression of channels suggests the possibility that those proteins could be able to be used as tumour markers or as powerful tools for diagnosis.

Report of the PhD student participation

K_v1.3 and K_v1.5 in cancer models: glioblastoma and pancreatic ductal adenocarcinoma (PDAC).

Albert Vallejo Gracia performed all the experiments and the data analysis of this article except the immunohistochemistry and immunocytochemistry studies.

Dr. Antonio Felipe Campo
Thesis director

K_v1.3 and K_v1.5 role in other cancer models: glioblastoma and pancreatic ductal adenocarcinoma (PDAC).

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SUMMARY

Membrane ion channels participate in the control of cell proliferation and some K_v channels seem to be related with cancer development. Evidence suggests that tumour cells contain an aberrant expression of K_v channels. Because K_v1.3 and K_v1.5 remodelled during tumorigenesis, the objective of this work was to analyse their expression in human brain and pancreas tumours. We analyse a potential role of both voltage dependent potassium channels in human glioblastoma and pancreatic ductal adenocarcinoma cells. We wondered whether K_v1.3 or K_v1.5 expression correlated with malignancy or tumour aggressiveness. Our studies indicated that while K_v1.3 and K_v1.5 channels are both present in human glioblastoma samples only K_v1.5 was clearly expressed in PDAC cell lines. Kv1.5 seems not to be playing a notable role either in proliferation or migration in PDAC. General pharmacological tools other than the specific Psora-4, as 4-AP, opened the possibility that other K_v1 channels, rather than K_v1.3 and K_v1.5, could be involved. However, the anomalous expression of Kv1.3 and Kv1.5 suggests the possibility that those channels could be used as tumour markers for diagnosis.

INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Current technologies like genomics and proteomics have increased the number of human genes known to be differentially expressed in normal and malignant tissues. This new perspective shows that potassium channels could play a relevant role in cancer therapy. Potassium channels are one of the most diverse and spread membrane protein families and they are codified by more than 75 different genes in humans. Voltage dependent potassium channels (K_v), a superfamily compound by 12 families (K_v1- K_v12), play a clear and relevant role in excitable tissues like neurons or muscles, taking part in the maintenance of membrane potential and the control of action potentials. In non-excitable cells, K_v channels also contribute to a wide variety of biological processes including the maintenance of the smooth muscle tone, cell growth, cell volume regulation, adhesion, motility, epithelial transport, insulin release and apoptosis. Several studies demonstrate that some members of the K_v1 family, like K_v1.3 or K_v1.5, are involved

in cell growth. $K_v1.3$ was the first channel reported to modulate cell proliferation and is crucial for activation and IL-2 production in G_1 phase and progression through the cell cycle in T lymphocytes. $K_v1.3$ expression also modulates cell proliferation in vascular smooth muscle cells, macrophages, microglia, oligodendrocyte progenitors and several cancer cell types (Villalonga, et al. 2010; Jiménez-Pérez et al. 2015; Pardo 2004). On the other hand, several studies suggest that $K_v1.5$, the predominantly cardiac channel, acts in astrocytes, oligodendrocytes, microglia, macrophages and myoblast proliferation (Villalonga, et al. 2010; Attali et al. 1997; Kotecha and Schlichter 1999; Macfarlane and Sontheimer 2000; Villalonga et al. 2008). $K_v1.3$ and $K_v1.5$ expression in tumour cells, their role in their pathophysiology and the fact that their antagonists arrest proliferation have attracted researchers attention (Comes et al. 2013; Felipe et al. 2006).

Malignant gliomas are considered brain tumours because initiate either from brain or spinal cord glial cells. Gliomas represent the 30 % of brain tumours and are classified according to their grade or pathologic evaluation status: low- (benign) and high-grade (malign), or according to the type of glial cell involved in the tumour such as astrocytoma, ependymoma, oligodendroglioma or mixed. Glioblastoma, also known as glioblastoma multiforme (GBM) or grade IV astrocytoma is a type of astrocytoma rarely curable (length of survival of 12 to 15 months following diagnosis) because of the bad prognosis and the diffuse nature of glioblastoma migrating cells to apoptosis (Young et al. 2015; Le Calvé et al. 2010; Louis 2006). Preußat and co-workers have reported that differential $K_v1.3$ and Kv1.5 expression occurs with respect to the malignancy grade of the glioma tumour. Although $K_v1.5$ expression is elevated in astrocytomas, moderate in oligodendrogliomas, and low in glioblastomas, no such correlation is evident for $K_v1.3$. These data suggest that reduced levels of $K_v1.5$ protein in biopsies when compared to the levels found in adjacent healthy tissues may be a good biomarker candidate for both glioma detection and outcome prediction (Preußat et al. 2003). Other studies reveal abundant $K_v1.3$ and $K_v1.5$ expression in brain tumours and suggest that while $K_v1.3$ expression is notable in astrocytomas, $K_v1.5$ expression is elevated in glioblastomas (Bielanska et al. 2009). Apart from the controversy around whether $K_v1.3$ and $K_v1.5$ expression is increased in gliomas or in healthy cells, $K_v1.5$ expression also occurred more in diffuse astrocytoma than in high grade ones. Moreover, glioblastoma patients with higher $K_v1.5$ expression had slightly better survival (Comes et al. 2013; Macfarlane and Sontheimer 2000).

Pancreatic ductal adenocarcinoma (PDAC) is a malign neoplasm originally from transformed cells that come from pancreatic cancer. PDAC is the most lethal common cancer because it is usually diagnosed at an advanced stage and is resistant to therapy. The most common type of pancreatic cancer, around 95 % of those tumours, is the adenocarcinoma coming from the exocrine part of the pancreas. Signs and symptoms that will finally allow the diagnosis depend on the localization, size, type of tumour tissue and they can include abdominal pain, low back pain and jaundice. This cancer has an extremely poor prognosis, especially for patients with metastasis (80 % of all cases) where the surviving tax is 15 % and the relative survival is from 6 to 10 months (Ryan, et al. 2014). Recently, a wide analysis of $K_v1.3$ and $K_v1.5$ protein expression in a plethora of human tumours has been performed in our research laboratory. Results indicate that the wide majority of tumours experiment an alteration in the K_v channels expression. While $K_v1.3$ expression is low in the pancreas, $K_v1.5$ is quite abundant. Unlike to $K_v1.3$, $K_v1.5$ expression increases in most of human tumours. Thus, pancreatic neoplasms express more $K_v1.5$ than healthy specimens (Comes et al. 2013; Felipe et al. 2012; Bielanska et al. 2009).

In collaboration with Dr. Robert Kiss (Université libre de Bruxelles, Brussels) and Dr. L.A. Pardo (Max Planck Institute for Experimental Medicine, Göttingen, Germany) brain tumours and pancreatic cancer were analysed. The aim of our studies was to analyse the potential role and implication of the voltage dependent potassium channels $K_v1.3$ or $K_v1.5$ in human glioblastoma and pancreatic tumours. We wondered whether $K_v1.3$ or $K_v1.5$ protein expression correlated with malignancy degree and tumour aggressiveness. In both cases, our results demonstrated the expression of K_v channels in human glioma/glioblastoma and PDAC tumours. However, apparently no correlation between $K_v1.3$ and $K_v1.5$ expression and tumour malignancy grade was observed.

MATERIALS AND METHODS

Cell culture

The human U87 (ATCC code HTB-14) astrocytoma, U373 (ATCC code HTB-17) glioblastoma, U251 glioblastoma, Hs683 (ATCC code HTB-138) glioma and T98G (ATCC code CRL1690) glioblastoma multiforme cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Kiss's laboratory as detailed previously (Le Calvé et al. 2010). Xenografts for immunohistochemistry studies from MDA-MB-435S, U87G, G112, GBM and AsPC1 cell lines were kindly given by Pardo's laboratory. Regarding pancreatic cancer experiments, several immortalized cell lines coming from pancreatic cancers have been used: AsPC1, BxPC3, Capan1, MiaPaCa2 and Panc1, which present different proliferative rates and tumour malignancy (Table 1). Cells were maintained at 5 % CO_2 in a humidified atmosphere at 37 °C.

Cell line	Doubling time (h)	Culture media
AsPC1	38-40	RPMI + FCS 10%
BxPC3	48-60	RPMI + FCS 10%
Capan1	50-100	IMDM + FCS 20%
Panc1	52	DMEM + FCS 10%
MiaPaCa2	40	DMEM +FCS10% +HS2,5%

Table 1. Characteristics of pancreatic cell lines.

Reverse transcription of total mRNA and qRT-PCR

Total mRNA was isolated from cell lines using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total mRNA was reverse transcribed using SuperScrip® III First-strand synthesis system (Invitrogen). Finally, cDNA was diluted in water to a final volume of 25 µL. Reverse transcribed cDNA was taken as a template for qRT- PCR in order to determine gene expression. qRT-PCR was performed using specific TaqMan sets in a LightCycler 480 (Roche Applied Science) detection system.

Protein extraction and western blot analysis

Protein content of the human glioma homogenates was measured using the Bio-Rad Protein Assay. Protein samples (50 µg) and immunoprecipitates were boiled in Laemmli SDS loading buffer and separated by 10% SDS-PAGE. Next, the samples were transferred to nitrocellulose membranes (Immobilon-P, Millipore) and blocked in 5% dry milk supplemented with 0.05% Tween 20 in PBS before the immunoreaction. The filters were then immunoblotted overnight at 4 ºC with antibodies against $K_v1.3$ (1/500 Alomone, Rabbit Polyclonal), $K_v1.5$ (1/500 Alomone, Rabbit/Polyclonal) and β-actin (1/50000 Sigma, Mouse/Monoclonal). Then, a HRP conjugated secondary antibody was incubated with the filters before applying an ECL-chemioluminiscence protocol. Ramos B-cell and HEK 293 cells were used as positive (CT+) and negative (CT-) controls respectively. For imaging processing analysis FIJI software was used.

Pancreatic cells were harvested by trypsinization and washed once with PBS. After centrifugation, the cell pellet was resuspended and incubated for 30 minutes in non-denaturating lysis buffer containing 1 % Triton X-100, 50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA and protease inhibitor cocktail (Roche). The cell lysate was then centrifuged for 15 minutes at 16000 x*g*, the supernatant was recovered and the protein concentration was measured using BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Samples were loaded into 8-12 % NuPAGE Novex Bix-Tris Mini Gels (Invitrogen). Once the proteins were separated by SDS-PAGE, a voltage gradient was applied from 10 V to 50 V with stepwise increments of 10 V every ten minutes and the final step at 50 V for 30 min. Then, the membrane was rinsed with deionized water and dried for 2 hours at room temperature. The membrane was rehydrated with deionized water and treated with Blot signal enhancer (Thermo Scientific) according to the manufacturer's instructions. The membrane was then incubated for 1 hour in blocking buffer containing casein and TBS-0.05% Tween 20, followed by overnight incubation at 4 °C with primary antibody diluted in blocking buffer. Antibodies against K_v1.3 (1/500, Rabbit, Antibodies BCN; or 1/500, Mouse, Neuromab), K_v1.5 (1/500, Mouse, Alomone) or actin (1/1000, Goat, Santa Cruz) were used. After 5 washes with deionized water and 5 minutes incubation with TBS-T, the membrane was incubated for 45 minutes with horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking buffer. Secondary antibodies ECL Anti-Rabbit IgG-HRP (1/10,000, Donkey, GE Healthcare), ECL Anti-Mouse IgG-HRP (1/10,000, Sheep, GE Healthcare) and Anti-Goat IgG (H+L)-HRP (1/10,000, Rabbit, BioRad) were used. Next the membrane was washed 7 times with deionized water and incubated for 5 minutes with TBS-T. At last, the chemiluminescent HRP substrate (Millipore) was applied to the blot for 5 minutes, leading to light emission. For imaging processing analysis FIJI software was used.

Immunocytochemistry

Cells were plated on poly-L-lysine coated coverslips and fixed using 10 % formalin solution (Sigma) at 4 °C for 10 minutes, washed 3 times with PBS, and permeabilized with 0.5 % Triton X-100 (Sigma) in PBS for 5 minutes. Afterwards, cells were washed 3 times with PBST (0.05 % Tween 20 (Sigma) in PBS), blocked with 10 % BSA (Sigma) in PBST for 1 hour, and incubated with primary antibodies against K_v1.3 (1/200, Rabbit, Alomone) and K_v1.5 (1/500, Rabbit, Alomone; or 1/1000, Rabbit, Abcam) diluted in blocking solution overnight at 4 °C.

Cells were then rinsed 3 times, incubated for 1 hour at room temperature with secondary antibodies Alexa Fluor 488 anti-rabbit IgG (H+L) (1/1000, Goat, Invitrogen) diluted in blocking solution (Table 3), and washed again 3 times. Finally, the nuclei were counter-stained with 1:1000 dilution of TO-PRO-3 (Invitrogen) in PBST, the coverslips were mounted on a glass slide using Prolong Gold antifade reagent with DAPI (Invitrogen). Confocal images were taken and imaging processing was done using the image analysis software FIJI.

Immunohistochemistry

Tissue sections were deparaffined using xylene and rehydrated by decreasing ethanol dilutions (from 100 % to 90 % to 70 % to 0 %) for 5 minutes each step. Afterwards, the tissue sections were incubated for 30 minutes at 90 °C in 10 mM citrate buffer for antigen retrieval. Then, the sections were allowed to cool down for 1 h at room temperature, and washed with 0.05 % Tween 20 (Sigma) in TBS. Blocking was performed using 10 % BSA (Sigma) diluted in TBS and 0.05 % Tween 20 (Sigma). The tissue sections were incubated overnight at 4 °C with primary antibody (anti- K_v1.3 (1:200) and anti- K_v1.5 (1:500)

polyclonal antibodies, Alomone Labs, or anti-K_v1.5 (1:1000) polyclonal antibodies, Abcam) diluted in blocking solution, washed 3 times, and incubated for 1 hour at room temperature with secondary antibody (Alexa Fluor 488 anti-rabbit IgG (H+L) (1:1000) and Alexa Fluor 546 anti-mouse IgG (H+L) Goat/Polyclonal antibodies (Invitrogen)) diluted in blocking solution. After washing, the tissue sections were incubated with 1 µL of TO-PRO-3 (Invitrogen) diluted in 1 mL of blocking solution to counter- stain nuclei. Confocal images were taken and image processing was done using the image analysis software FIJI.

Proliferation and migration assays

Optimal conditions were set up in the IncuCyte™ machinery to study proliferative and migration (scratch-wound) processes. In both cases, foetal calf serum addition was the positive control of proliferation (+FCS) while the negative control was medium without serum (-FCS). We also used specific drugs to the different analysed proteins to test whether their block generate changes. 4-aminopyridine (4-AP) is a compound that inhibits non-specifically all types of Shaker potassium channels (K_v1). On the other hand, Psora-4 is a highly specific drug against both K_v1.3 (EC_{50}:3 nM) and K_v1.5 (EC_{50}: 7.7 nM) exhibiting 17- to 70-fold selectivity over closely related K_v1-family channels (K_v1.1, K_v1.2, K_v1.4, and K_v1.7). Psora-4 has no effects on human ether-a-go-go-related channel, K_v3.1, the calcium-activated K^+ channels (IKCa1, SK1-SK3, and BKCa), or the neuronal Na_v1.2 channel (Vennekamp et al. 2004).

Proliferation studies have been performed using two different approaches (Fig. 1). First, 10.000 – 50.000 cells/well were seeded in 24-well cells and were incubated 24 h with minimal medium or starving (-FCS) before the readdition of the FCS and the different conditions and drugs. By removing FCS for 24 hours, proliferation was stopped and cell population was homogenized at the same cell cycle phase (G_1). Alternatively, 1.000 – 5.000 cells/well were seed in the different 96-well plates and different drugs were added. After recording the data of the area occupied by cells or phase object confluence per unit of time by the IncuCyte™, those variables were plotted and the proliferation rate was calculated from the slope for each condition (in the starved experiments, the first 25 hours were not considered). Finally, we relativized every slope to the positive control for proliferation (10% FCS or similar). Consequently, the steeper the slope, the faster the proliferation (Fig. 1).

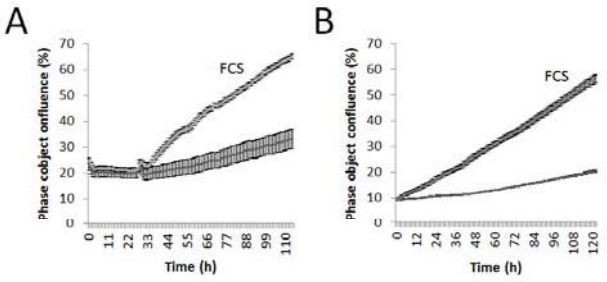

Figure 1. Proliferation in pancreatic cells lines. Representation of the two different approaches analysing proliferation in the presence (FCS 10%) or the absence of serum (Starving). In (A), but not in (B) cells were starved during the first 24 hours.

Electrophysiology

Cells were grown for 24 h on poly-L-lysine-coated glass coverslips. Macroscopic currents were recorded in the whole-cell configuration of the patch-clamp technique using an EPC-9 amplifier (HEKA). Patch pipettes with a tip resistance of 2–5 MΩ were made from Corning #0010 capillary glass (WPI). Series resistance was compensated by 70%. Internal solution contained (in mM); 120 KCl, 1 CaCl2, 2 MgCl2, 10 HEPES/HCl, pH 7.3 and the external solution contained (in mM); 160 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 8 glucose, 10 HEPES/NaOH, pH 7.4. The holding potential for all experiments was – 70 mV. To evoke voltage-gated currents, all cells were stimulated with 250-millisecond square pulses ranging from –60 to +100 mV in 20 mV steps. To analyse cumulative inactivation, currents were elicited by a train of 15 depolarizing voltage

steps of 200 milliseconds to +60 mV once every 400 milliseconds. Data processing and curve fitting were performed with Igor Pro (WaveMetrics). All recordings were performed at room temperature.

RESULTS

Immunohistochemistry of Kv1.3 and Kv1.5 in glioblastoma and pancreatic tumours

The voltage-dependent potassium channels $K_v1.3$ and $K_v1.5$ are expressed in numerous tissues playing a role in cellular proliferation (Comes et al. 2013). In addition, $K_v1.5$ can form heterotetramers with $K_v1.3$ and, via this interaction, $K_v1.5$ fines tune physiological responses (Villalonga et al. 2010). We first performed a $K_v1.3$ and $K_v1.5$ protein expression analysis in a number of human tumours. Different cancer cells from different histological type (MDA-MB-435S for melanocyte/melanoma, U87G for glioma/astrocytoma, G112 for glioma, GBM for glioblastoma multiforme and AsPC1 for pancreatic cancer) were injected in mice, and their xenografts were stained by immunohistochemistry. We found no correlation between the channel expression and the type of tumour. Fig. 1 shows that $K_v1.3$ staining was evident in MDA-MB-435S, U87 and AsPC1 cells xenograft, where $K_v1.5$ was abundant as well. Pancreatic cancer AsPC1 cells presented the clearer and stronger $K_v1.3$ and $K_v1.5$ labelling.

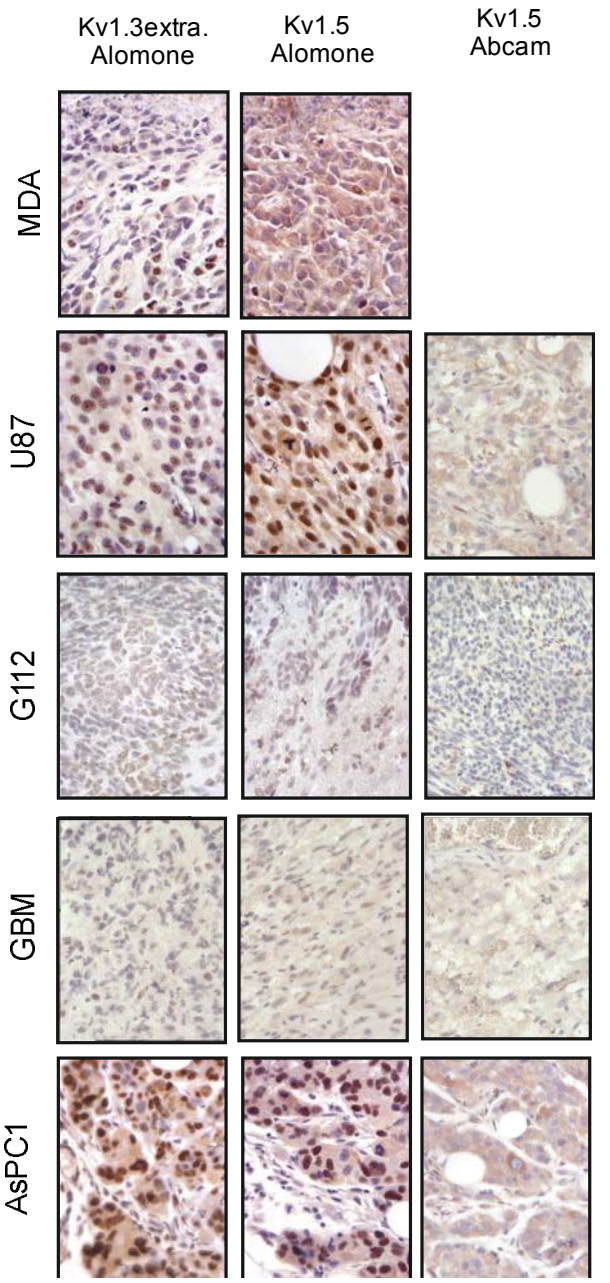

Figure 2. $K_v1.3$ and $K_v1.5$ in tumour samples. Immunohistochemistry images from xenografts of MDA-MB-435S (melanocyte/melanoma), U87G (astrocytoma), G112 (glioma), GBM (glioblastoma multiforme) and AsPC1 (pancreatic cancer) cells stained with different $K_v1.3$ and $K_v1.5$ antibodies.

K_v1.3 and K_v1.5 abundance in human glioblastoma tumours

To further analyse the expression of K_v1.3 and K_v1.5 in glioma tumours, we performed a parallel screening of human glioblastomas analysed by western blot. Again K_v1.3 and K_v1.5 were detected in human glioblastoma samples. Three different western blots were performed and several samples were used as internal controls. Therefore, results in Fig 3B, represent the relative abundance of K_v1.3 and K_v1.5 taking into account: (i) the abundance of β-actin and (ii) the relative expression of both channels in the U87 sample. Data in Fig. 3 indicates that the expression of K_v1.3 and K_v1.5 was heterogeneous among the different samples and types of glioblastomas.

As an anomalous expression of K_v1.3 and K_v1.5 has been observed in various types of tumours (Bielanska et al. 2009), we wondered whether any relationship between K_v channel expression and glioblastoma malignancy or invasiveness existed. Previous studies suggested that K_v1.5 expression inversely correlates with glioma aggressiveness. However, the data, analysed in R. Kiss's laboratory, did not present such correlation between K_v1.5 and K_v1.3 expression with glioma malignancy (personal communication). Due to the fact that our group has previously described how K_v1.5 inhibits myoblast cell growth through a mechanism involving the increase of the $p27^{kip1}$ cyclin-dependent kinase inhibitor (Villalonga et al. 2008) and because, in the literature, $p27^{kip1}$ expression in human glioma immortalized cell lines is described to inversely correlate with proliferation and Ki-67 marker expression (Fuse et al. 2000), we plotted our channel protein expression data against $p27^{kip1}$ for several glioblastoma cell lines. However, again no correlation was observed (data not shown). Thus, concurrent and independently to Kiss's lab, we dismissed any relationship between the studied K_v channels and tumour malignancy.

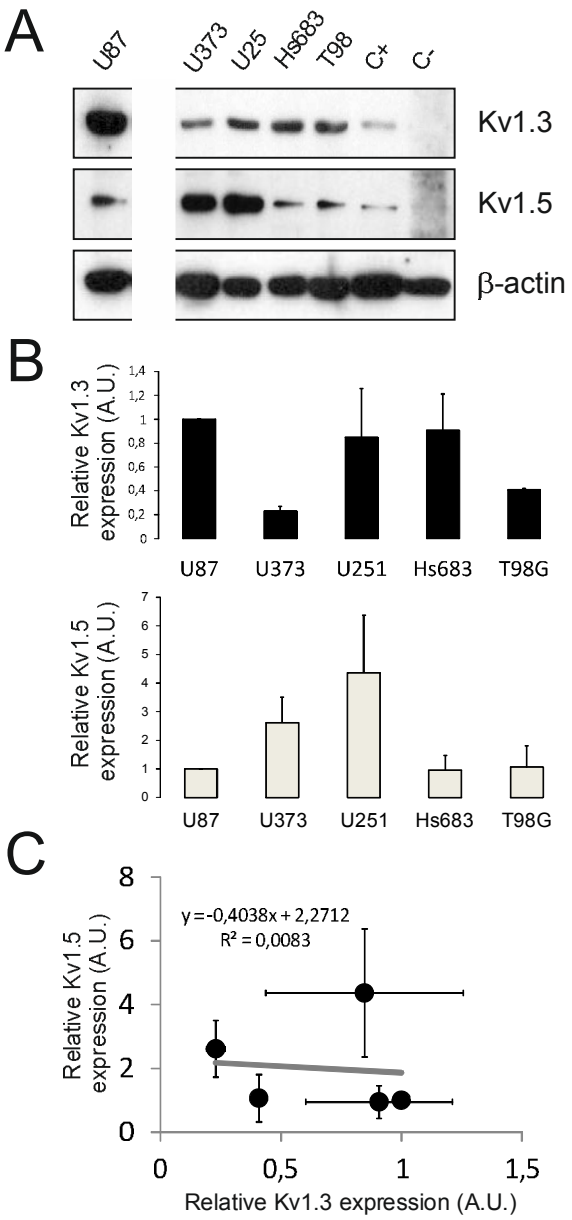

Figure 3. Expression of K_v1.3 and K_v1.5 in glioblastoma samples. (A) Representative western blot (n=3). β-actin was used as a loading control. Ramos B-cells were used as positive control whereas HEK 293 cells were used as negative controls. (B) Diagrams showing relative expression of the channels. (C) Plot of K_v1.3 versus K_v1.5 protein expression.

K_v channel expression in PDAC

The role of K_v1.3 and K_v1.5 channels in pancreatic cancer had not been previously reported. Therefore, we first undertook a characterization of K_v1.3 and K_v1.5 in different pancreatic cell lines. We analysed K_v1.3 and K_v1.5 protein expression

by western blot in the different pancreatic cancer cell lines (data not shown). K_v1.3 and K_v1.5 were differentially expressed in all cell types. Concerning K_v1.5, AsPC1 and MiaPaCa2 cell lines expressed the channel to a higher extent than the other pancreatic cell lines. This data was further confirmed by immunocytochemistry. K_v1.5 channel showed an intracellular retention, typical from this channel (Fig. 4).

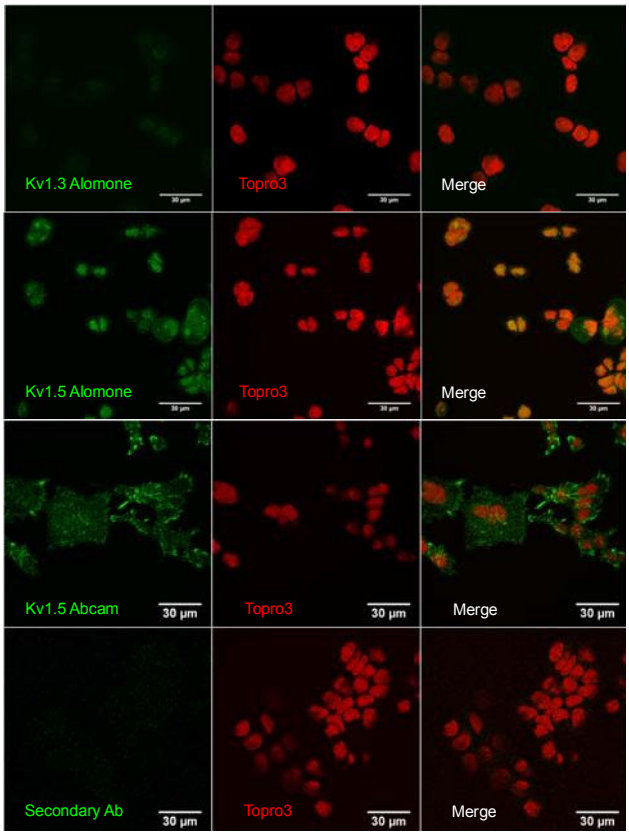

Figure 4. Immunocytochemistry of K_v1.3 and K_v1.5 in AsPC1 cells. Different antibodies were used. Nuclei were also stained in middle panels. Right columns represent merge. Bottom panels, negative control of secondary antibody.

Pancreatic cancer cell lines exhibit outward K_v currents

Electrophysiological studies were performed to analyse K_v currents in different pancreatic cell lines. Current-voltage relationships and ramp protocols (Fig. 5) identified K^+ currents in pancreatic cancer cell lines. Although outward K^+ currents were evoked in AsPC1 cells (Fig. 5 A-B), we could not clearly elucidate whether K_v1.3 or K_v1.5 were the main responsibles of those currents. The shift between the theorical voltage activation thresholds of K_v1.3 or K_v1.5 and the ones observed to more positive values in current-voltage relationships of those cell lines presented an obvious discrepancy. On the other hand, K_v1.3 undergoes cumulative inactivation upon repeated depolarization. In AsPC1 and MiaPaCa2 cell lines, K_v1.3 presence is questioned because K_v currents showed a negligible cumulative inactivation (Fig. 5C). Although they exhibited outward K_v currents (Fig. 5D), voltage-ramps in Fig. 5E indicated, again, that voltage-gated outward currents are activated at membrane potentials more depolarized than -30 mV or -10 mV. It is worth to mention that AsPC1 and MiaPaCa2, notable expressed K_v1.5 instead .

K_v channels in proliferation and migration of pancreatic cancer cell lines

Proliferation assays were performed to analyse the potential implication of K_v1.3 and K_v1.5 channels during cell proliferation and migration. Cells were incubated in the presence of serum (FCS), with or without a prior 24 h starvation. As you can see in Fig. 6, the difference between positive control (10 % FCS) and negative control (starving) were notable. The addition of the different compounds (Psora-4, 4-AP and DMSO) impaired proliferation in both approaches (Fig. 6 A, B). By using this combination of toxins and chemicals we demonstrated that K_v channels were involved in proliferation (Fig. 6). The 4-AP, a non-specific K_v blocker, inhibited proliferation with a semi-maximal inhibition (EC_{50}) of approximately 1.5 mM (Fig. 6C). We next used more selective compounds. Thus, Psora-4, a highly specific drug for K_v1.3 and K_v1.5, inhibited cell proliferation (Fig. 6) and this was not a consequence of the DMSO (vehicle).

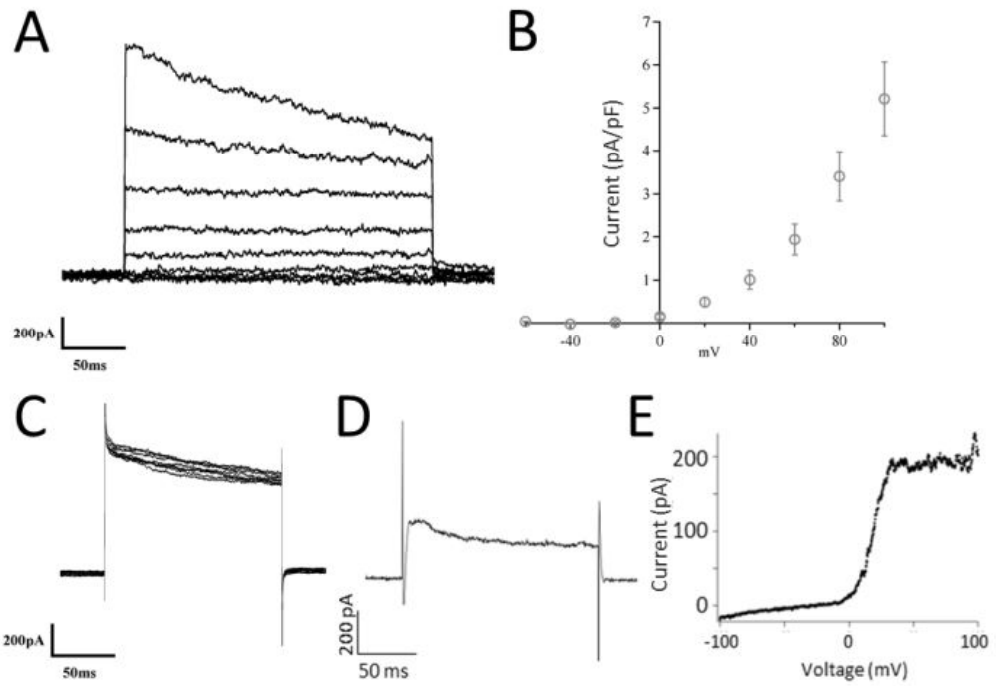

Figure 5. Biophysical properties of pancreatic cell lines. (A) Representative current traces obtained from AsPC1 cells. (B) Current density (pA/pF) plotted against voltage (mV). (n=6). (C) Representative traces for cummulative inactivation of outward K^+ currents in AsPC1. (D) Representative outward K^+ current and (E) ramp obtained from MiaPaCa2 cells.

To further analyse the potential physiological role of $K_v1.3$ and $K_v1.5$ during pancreatic cancer cell proliferation a number of cell types were evaluated. Most pancreatic cancer cell models arrest their proliferation in the absence of FCS (Table 5). Proliferation was generally blocked a relatively low 4-AP doses (2 mM), suggesting a putative role of K_v channels. Surprinsingly, Psora-4 did not arrest proliferation. However, high drug concentrations (5 µM) were able to block proliferation in AsPC1 (Fig. 6B). Dose-effect studies determine the minimum inhibitory concentrations for K_v blockers. They were, approximately 2 mM for 4-AP and 5 µM for Psora-4. The putative role of $K_v1.3$ and $K_v1.5$ in pancreatic cancer cell proliferation seemed minor, as doses of Psora-4 that were tenfold higher than the IC_{50} triggered no effect. In summary, pharmacological experiments provide evidence that K_v channels are involved in pancreatic cell proliferation, although $K_v1.3$ and $K_v1.5$ do not play an apparent role.

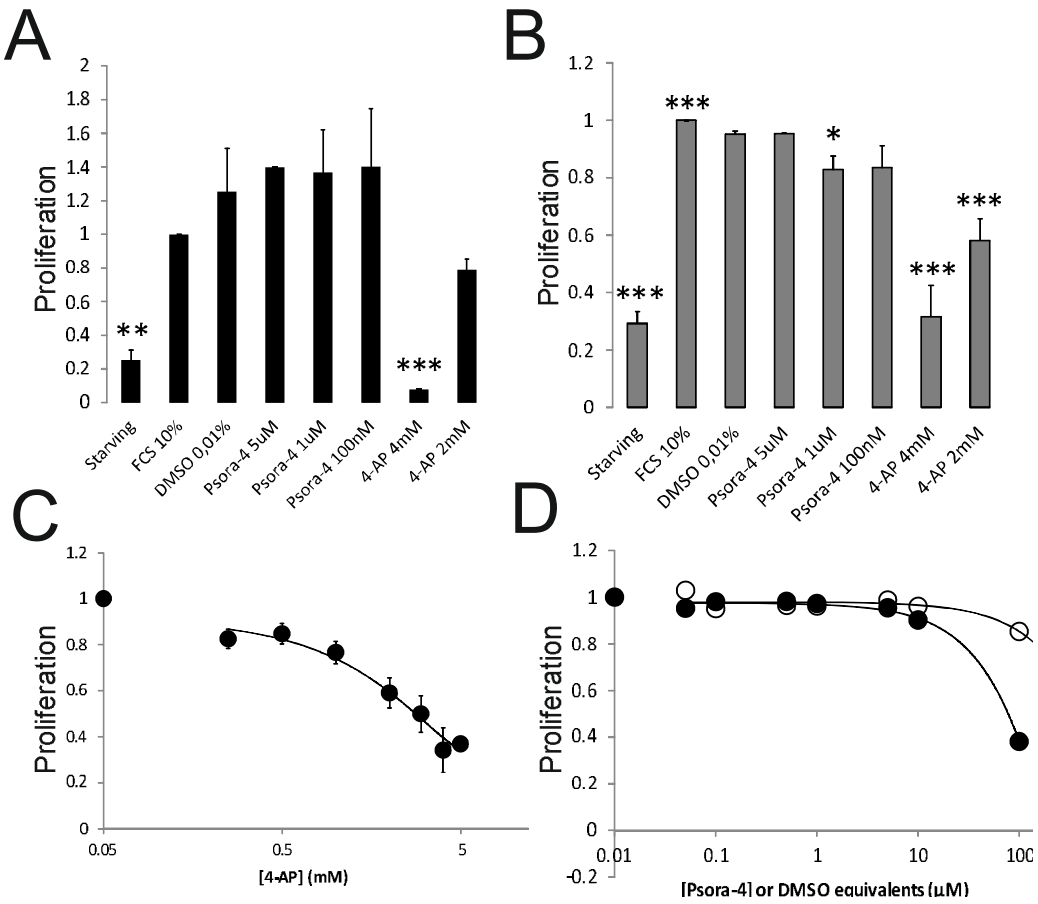

Figure 6. Proliferation of AsPC1 cells. (A) Proliferation (relative phase object confluence) of starved AsPC1 cells (n = 3). (B) Proliferation (relative phase object confluence) of non-starved AsPC1 cells (n = 6). The slope of the FCS-dependent proliferation was taken as reference and compared with the rest. Mean ± SEM is represented. (**P*<0.05; ***P*<0.01; ****P*<0.001 vs DMSO; Student's *t*-test) (C) Relative percentage of proliferation (%) in dose-effect responses (non-starved approach, n=3) for 4-AP. (D) Relative percentage of proliferation (%) in dose-effect responses experiments (non-starved approach, n=2) for Psora-4 (black circles) and DMSO (white circles). Mean ± SEM is represented.

Because no clear relation was observed with proliferation, we wanted to study the potential channels involvement in migration phenomena. $K_v1.3$ is involved in macrophage migration (Villalonga et al. 2010);. Due to that cell migration is one of the key features of neoplastic cell growth and invasion, we studied the migration capacity of Panc-1 cell line. However, neither Psora-4 nor 4-AP produced any significant effect (data not shown).

	Starved	n	Analysed period (days)	Starving (0%)	FCS (10%)	DMSO	Psora-4		4-AP	
						0.01%	1µM	100nM	4mM	2mM
AsPC1	+	3	4	0.25±0.03 **	1	1.25±0.26	1.37±0.25	1.40±0.60	0.08 ***	0.08±0.06 ***
	-	6	4	0.29±0.04 ***	1 ***	0.95±0.01	0.83±0.12 *	0.83±0.08	0.32±0.1 ***	0.58±0.07 ***
BxPC3	+	1	3	-0.16±0.01	1±0.02	1.07±0.04	0.94±0.05	1.01±0.05	---	0.91±0.06
	-	1	3	-0.04±0.02	1±0.10	1.09±0.07	0.72±0.08	0.97±0.09	-0.15±0.04	0.92±0.15
Capan1	+	2	4	0.06±0.01 **	1	1.12±0.08	1.29±0.07	1.21±0.02	-0.02	0.13
	-	2	4	0.27±0.08 *	1	0.82±0.13	0.82±0.2	0.65±0.17	-0.01±0.02 *	0.16±0.01 *
Panc1	+	1	3	0.43±0.02	1±0.22	0.97±0.19	0.84±0.09	0.82±0.05	---	0.68±0.20
	-	2	3	0.58±0.13	1	0.89±0.16	0.89±0.01	1.08±0.07	0.35±0.23 *	0.68
MiaPaCa2	-	1	4	0.51±0.16	1±0.27	0.93±0.23	0.87±0.13	0.92±0.11	---	---

Table 5. Proliferation assays in pancreatic cell lines. Relative percentage of proliferation of pancreatic cell lines under different insults. When n=1, it is represented mean ± SD (from intra-experiment replicates). When n>1 values are mean ± SEM; **P*<0.05; ***P*<0.01; ****P*<0.001 vs DMSO; Student's *t*-test.

DISCUSSION

Ion channels participate in the control of cell proliferation and some K_v channels seem to be related with cancer development. Several studies demonstrated that channels antagonists regulate cell proliferation. However, the specific mechanism remains unknown. In addition, evidence suggests that tumour cells, which dysregulate their proliferation and differentiation, contain an altered expression of K_v channels (Felipe et al. 2012). Because $K_v1.3$ and $K_v1.5$ remodelled during tumorigenesis, the objective of this work was to analyse the expression of both channels in human glioblastomas and pancreatic ductal adenocarcinomas.

Our studies indicate that $K_v1.3$ and $K_v1.5$ channels are both present in human glioblastoma samples. This data agrees with previous studies where the existence of those channels in human gliomas was proposed (Preußat et al. 2003). Although we had previously described that myoblastic proliferation is mediated by $K_v1.5$ expression through a mechanism that involves cyclin-dependent kinase inhibitors $p21^{cip-1}$ and $p27^{kip1}$ (Villalonga et al. 2008), we did not find any correlation between $K_v1.3$ and $K_v1.5$ channel expression and tumour malignancy by plotting them against $p27^{Kip1}$ expression, which inversely correlate with tumour proliferation (Fuse et al. 2000). The importance of K_v channels in glial cell physiology is further supported by the presence of $K_v\beta$ ancillary subunits (Pongs and Schwarz 2010) and also by the fact that $K_v1.3$ in microglia is reported to contribute to the respiratory burst in these cells and microglia-mediated neuronal killing (Chi et al. 2012). Therefore, further research must be performed.

In addition, K_v channels are expressed in pancreatic tumours, but there are no reports addressing their possible role during physiological processes such as cell proliferation and invasiveness (Comes et al. 2013; Bielanska et al. 2009). Our work confirms K_v channels expression,

especially K_v1.5, in human pancreatic cancer cells. However, they did not seem to be playing a pivotal role in proliferation or migration of our pancreatic cancer model. The use of general pharmacological tools such as 4-AP, instead specific Psora-4, opens the possibility that other K_v1 or Shaker channels, rather than the K_v1.3 and K_v1.5, could be involved in those processes.

In summary, our data indicates that K_v channels are expressed in different human tumours such as glioblastomas or pancreatic ductal adenocarcinoma. While K_v1.3 and K_v1.5 are detected in several glioblastoma cell lines, K_v1.5 channel seems the predominant one in pancreatic cancer cell lines. The altered expression of both channels suggests the possibility that those proteins could be able to be used as tumour markers or as powerful tools for diagnosis. Furthermore, it is tempting to speculate that in addition to their well-described function in regulating excitability, K_v-driven signalling pathways could be independent of ion flux but related to conformational changes (Kaczmarek 2006).

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3.2. CHAPTER 2. KCNE SUBUNITS IN THE LEUKOCYTE PHYSIOLOGY

3.2. KCNE subunits in the leukocyte physiology.

Published in Channels (Austin). Mar-Apr;7(2):85-96. 2013.

KCNE gene expression is dependent on the proliferation and mode of activation of leukocytes.

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SUMMARY

Leukocytes have a limited repertoire of Kv channels, whose physiological role is under intense investigation. A functional Kv channel is an oligomeric complex composed of pore-forming and ancillary subunits. The KCNE gene family is a novel group of modulatory Kv channel elements in leukocytes. Here, we characterized the gene expression of KCNEs (1-5) in leukocytes and investigated their regulation during leukocyte proliferation and mode of activation. Murine bone-marrow-derived macrophages, human Jurkat T-lymphocytes and human Raji B-cells were analyzed. KCNEs (1-5) are expressed in all leukocytes lineages. Most KCNE mRNAs show cell cycle-dependent regulation and are differentially regulated under specific insults. Our results further suggest a new and yet undefined physiological role for KCNE subunits in the immune system. Putative associations of these ancillary proteins with Kv channels would yield a wide variety of biophysically and pharmacologically distinct channels that fine-tune the immunological response.

Report of the PhD student participation in the article

KCNE gene expression is dependent on the proliferation and mode of activation of leukocytes.
Published in Channels (Austin), Impact Factor (2013): 2.317

Albert Vallejo Gracia performed all the cDNA synthesis, the KCNEs RT-PCR experiments (except for KCNE4) and RT-PCR analysis in Jurkat and Raji cell lines.

Dr. Antonio Felipe Campo
Thesis director

RESEARCH PAPER

Channels 7:2, 85–96; March/April 2013; © 2013 Landes Bioscience

KCNE gene expression is dependent on the proliferation and mode of activation of leukocytes

Laura Solé,[1] Albert Vallejo-Gracia,[1] Sara R. Roig,[1] Antonio Serrano-Albarrás,[1] Laura Marruecos,[2] Joan Manils,[2] Diana Gómez,[2] Concepció Soler[2] and Antonio Felipe[1,*]

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Keywords: voltage-dependent potassium channels, gene expression, macrophages, lymphocytes, cell-cycle, activation, regulatory subunits

Voltage-dependent K^+ (Kv) channels are tightly regulated during the immune system response. Leukocytes have a limited repertoire of Kv channels, whose physiological role is under intense investigation. A functional Kv channel is an oligomeric complex composed of pore-forming and ancillary subunits. The KCNE gene family is a novel group of modulatory Kv channel elements in leukocytes. Here, we characterized the gene expression of KCNEs (1–5) in leukocytes and investigated their regulation during leukocyte proliferation and mode of activation. Murine bone-marrow-derived macrophages, human Jurkat T-lymphocytes and human Raji B-cells were analyzed. KCNEs (1–5) are expressed in all leukocytes lineages. Most KCNE mRNAs show cell cycle-dependent regulation and are differentially regulated under specific insults. Our results further suggest a new and yet undefined physiological role for KCNE subunits in the immune system. Putative associations of these ancillary proteins with Kv channels would yield a wide variety of biophysically and pharmacologically distinct channels that fine-tune the immunological response.

Introduction

Voltage-dependent potassium (Kv) channels play an important role in determining resting membrane potential and controlling action potentials in nerves and muscles.[1] In addition, Kv channels control the proliferation and activation of macrophages and lymphocytes.[2,3] Leukocytes have a limited repertoire of Kv channels, which include Kv1.3, Kv1.5 and Kv11.1, which are major determinants of cell physiology.[4] While Kv1.3 is ubiquitous in the immune system, Kv1.5 functions primarily in mononuclear phagocytes and Kv11.1 in lymphoblastoid proliferative cells.[5-8] In addition, other Kv channels play minor roles and have also been detected in specific cell subtypes.[9,10] Many Kv channels can assemble promiscuously, yielding a wide variety of biophysically and pharmacologically distinct channels. Therefore, native currents are not necessarily recapitulated through the activity of a single subunit. Furthermore, the presence of regulatory subunits enhances this complexity.[11]

Kvβ regulatory subunits might modulate Kv1.3 and Kv1.5 channels.[11-13] These ancillary peptides are expressed in lymphocytes and macrophages and can form heteromeric structures.[12,13] Different *Kv*β*1*, *Kv*β*2* and *Kv*β*3*, generate Kvβ proteins that are differentially regulated through leukocyte proliferation and activation.[11] The association of different Kvβ subunits with Kv1.3 and Kv1.5 channels generates distinct functional Kv channel complexes that fine-tune the cellular response.[11-13] There has been a massive effort by the research community to characterize Kv channels; however, there are few studies concerning regulatory subunits in leukocytes.[11-13] The recent identification of novel modulatory proteins has attracted much attention.

KCNEs are a group of regulatory subunits composed of five-members (KCNE1–5).[14] KCNE peptides are single-span membrane proteins that modulate many Kv channels. Characterized interactions, such as Kv7.1 (KCNQ1)/KCNE1 and Kv11.1 (hERG)/KCNE2 channels, recapitulate the cardiac I_{ks} and I_{kr} currents, respectively.[14] Several KCNE mutations trigger severe cardiac channelopathies and demonstrate the pivotal role of these ancillary peptides in cardiovascular physiology. The implication of KCNE1–5 peptides in cardiac physiology is under intense investigation.[15] Although many KCNE subunits share tissue expression with Kv1 channels, their interactions need to be defined. Thus, Kv1.3 and KCNE1 were simultaneously cloned in human Jurkat T-cells.[16] It was shown that KCNE4 modulates Kv1.1 and Kv1.3 channels and their heteromeric forms.[17] Furthermore, an interaction between KCNE2 with Kv1.5 and Kv1.3 in murine ventricles and choroid plexus epithelium has been proposed.[18,19]

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Submitted: 10/05/12; Revised: 12/13/12; Accepted: 12/13/12
http://dx.doi.org/10.4161/chan.23258

Scarce information is available concerning the role of KCNE peptides in leukocytes. We recently demonstrated that KCNE4 associates with Kv1.3, suppressing K^+ currents through modulations in trafficking, surface expression and channel gating.[20] Although few preliminary screenings support a KCNE variable phenotype, there have been no KCNE regulation studies involving the immune system.[21] Therefore, the aim of the present work was to identify the presence of KCNE regulatory subunits in leukocytes and further characterize their expression during leukocyte proliferation and activation. We demonstrate that macrophages and T- and B-lymphocytes express KCNE subunits. In addition, the expression of many KCNE proteins is cell cycle dependent, and distinct cell-specific insults differentially regulate KCNE expression. In summary, leukocytes express KCNE regulatory subunits, and their expression varies with proliferation and activation; therefore, we suggest that the heteromeric Kv channel structure might be variable.

Results

Regulation of KCNE modulatory subunits through MCSF-dependent proliferation and LPS- and IFNγ-induced activation in macrophages. Macrophages perform critical functions in the immune system, acting as regulators of homeostasis and effector cells in infection, wounding, and tumor growth. In response to different growth factors and cytokines, macrophages proliferate and become activated or differentiated.[22] We used BMDM as a cell model that mimics the physiological schedule within the body. Mature cells might proliferate or become activated after the application of specific stimuli, such as MCSF, which is a specific growth factor for this cell type.[22] After incubation of BMDMs for 18–24 h in the absence of MCSF, cell growth was terminated (> 98%) and the cells became quiescent.[3,22] The addition of MCSF (1200 U/ml) triggered macrophage proliferation and an increase of the outward K^+ currents (**Fig. 1A and Fig. S1**).[3,12,22] Although BMDMs expressed all five KCNE subunits, different cell cycle-dependent patterns were observed (**Fig. 1B–G**). KCNE2, KCNE4 and KCNE5 notably increased at 6 h during the early phases (G1/S) of the cell cycle (**Fig. 1C, E and F**). In contrast, KCNE3 exhibited the opposite behavior (**Fig. 1D**), and KCNE1 levels remained constant throughout the cell cycle (**Fig. 1B**). Therefore, proliferation generated major differences in the KCNE phenotype (**Fig. 1G**).

The incubation of bone marrow macrophages with LPS and IFN-γ inhibits cell proliferation.[22,23] However, while LPS triggers apoptosis, IFN-γ protects the cells from programmed cell death.[23,24] **Figure 2** shows that after 24 h of IFN-γ-induced activation (300 U/ml), confirmed by iNOS abundance (**Fig. 2A**),[25] the expression of the KCNE subunits was relatively stable. Moreover, a gradual decrease of KCNE3 expression was also observed (**Fig. 2D**), while the other KCNEs experienced only minor changes in expression (**Fig. 2B–F**). Similar to proliferation, KCNE4 and KCNE5 were the most abundant subunits, and KCNE3 was the least abundant subunit (**Fig. 2G**).

The addition of 100 ng/ml LPS to proliferating macrophages elevated K^+ currents (**Fig. S1**), induced the expression of iNOS (**Fig. 3A**), blocks the cell cycle at the G1/S boundary and induces apoptosis.[23] Under this treatment, the expression of not only KCNE3 but also KCNE4 and apparently, but not significantly, KCNE2 was also reduced (**Fig. 3B–F**). However, the KCNE phenotype in LPS-treated macrophages was similar to that observed with IFN-γ. Thus, KCNE4 and KCNE5 were the most abundant subunits, whereas KCNE3 was least abundant (**Fig. 3G**).

KCNE regulatory subunits during the proliferation and LPS- and PMA-induced activation of Raji B-lymphocytes. Human B-lymphocytes express Kv1.3 and Kv11.1 channels that might be associated with KCNE subunits.[4] Raji B lymphocytes are a lymphoblast-like cell line derived from Burkitt's B-cell lymphoma. Although neoplastic proliferation increases the expression of Kv1.3 and Kv11.1, no information is available concerning the KCNE subunits.[8] Unlike macrophages, activated B cells undergo proliferation and differentiation into memory and plasma cells. We first analyzed the expression of KCNE subunits in resting Raji B-lymphocytes. The abundance of KCNE subunits in B-cells starved for 24 h indicated that the expression of KCNE1 and KCNE4 was extremely low (**Fig. 4G**). FBS-dependent proliferation triggered the differential regulation of KCNEs subunits (**Fig. 4A–F**). While KCNE2, KCNE3 and, to a lesser extent, KCNE4 showed decreased expression after 24 h, the expression of KCNE1 and KCNE5 remained constant (**Fig. 4B–F**). However, the KCNE profile in B cells throughout the cell cycle progression was mostly stable and after 24 h, the KCNE phenotype was recovered to that observed at the initial stage (**Fig. 4G**).

Raji B-lymphocytes, incubated with LPS (100 μg/ml), became activated as confirmed by the expression of TNFα (**Fig. 5A**).[26] However, after 24 h of LPS-induced activation, no major changes in the KCNE profile were observed (**Fig. 5B–G**). In addition, 10 nM PMA activation, which also elevated TNFα expression (**Fig. 6A**),[27] did not trigger apparent changes in KCNE expression (**Fig. 6B–F**). As a consequence, and similar to the results obtained with LPS-induced activation, no major differences in the KCNE profile were observed (**Fig. 6G**).

KCNE regulatory subunits during the proliferation and PMA/PHA-induced activation of Jurkat T-lymphocytes. Human T-lymphocytes expressing a limited repertoire of Kv1 channels, which include Kv1.1, Kv1.4, Kv1.6 and Kv1.3.[9,28] Kv1.3 and KCNE1, were isolated simultaneously in Jurkat T cells, and KCNE4 regulates Kv1.1 and Kv1.3.[16,17,20] Although we observed the expression of all KCNE transcripts in Jurkat T cells, surprisingly, KCNE1 was, by far, the less abundant KCNE subunit in Jurkat cells (**Fig. 7G**). Unlike KCNE1, KCNE3 and KCNE5, FBS-dependent proliferation (**Fig. 7A**) triggered a cell cycle-dependent increase of KCNE2 and KCNE4 expression (**Fig. 7B–F**). Compared with the relative abundance among KCNE subunits under FBS-dependent proliferation, the higher increase corresponded with KCNE4 expression. However, the KCNE profile experienced minor changes (**Fig. 7G**).

The combination of PHA (5 μg/ml) and PMA (80 nM) triggers an important activation of Jurkat T cells[28] reflected by the massive production of IL-2 (**Fig. 8A**). Under these circumstances, an important remodeling of KCNE mRNA expression

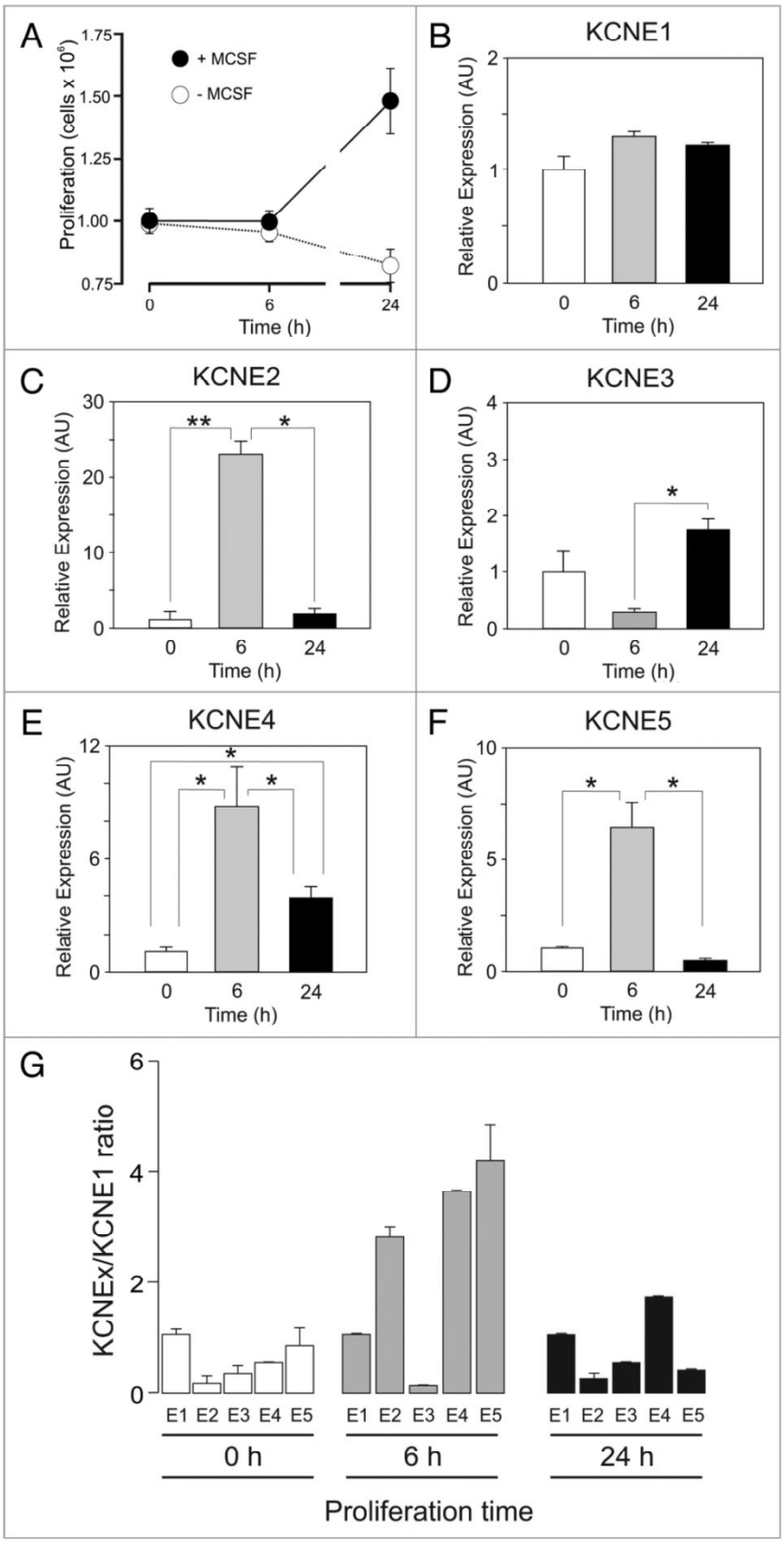

Figure 1. Expression and regulation of KCNE (1–5) mRNA in MCSF-dependent BMDM proliferation. G_0-arrested BMDMs were incubated in the presence of 1,200 U/ml MCSF, and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) MCSF-dependent proliferation of BMDM. The values are given as the mean ± SEM and represent cell number of four independent experiments each performed in triplicate. ●, +MCSF; ○, –MCSF. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). *, $p < 0.05$; **, $p < 0.01$ (Student's t-test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h of MCSF-dependent proliferation.

was observed (**Fig. 8B–G**). Again, KCNE1, the less abundant subunit, remained constant (**Fig. 8B and G**). However, the KCNE4 mRNA expression, which was initially low, showed an increase under PHA/PMA incubation (**Fig. 8E and G**). Moreover, the expression of KCNE5 and, to a lesser extent, KCNE3 steadily increased (**Fig. 8D and F**). In contrast, KCNE2 expression was significantly decreased (**Fig. 8C**). Our results demonstrated that the expression of KCNE4 was, by far, the major regulated under PMA/PHA-induced activation (**Fig. 8G**).

Discussion

Kv channels are involved in maintaining resting membrane potential and participate in the immune system response.[2,5] In nerve and muscle cells, native K^+ currents are not recapitulated through single-channel genes. Therefore, the hetero-oligomerization of related Kv subunits and their association with regulatory peptides to form functional channelosomes is crucial for cell physiology.[12,29] Leukocytes have a limited Kv repertoire that remains elusive. Kv channels in the immune system might be formed through associations between Kv products and auxiliary subunits, which enormously increase the K^+ current phenotype and fine-tune the immunological response.[12] Thus, Kv1.3 and Kv1.5, and Kv11.1 channels have been implicated as important targets in leukocyte physiology.[5,7,8,30] In addition, Kv1.1, Kv1.2, Kv1.6, Kv3.1 and Kv7.1 have also been detected in

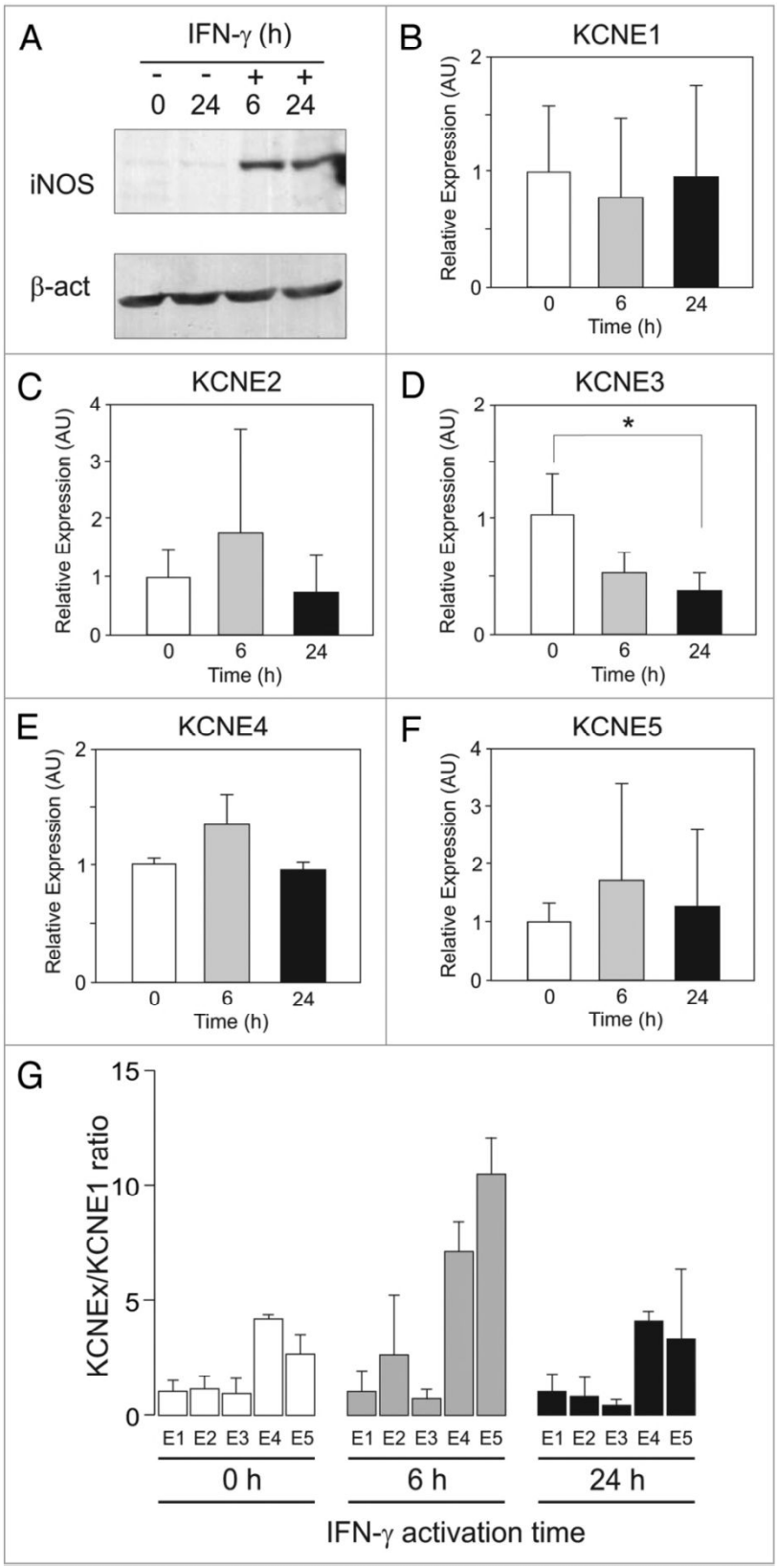

Figure 2. Expression and regulation of KCNE (1–5) mRNA in IFN-γ-activated BMDMs. BMDMs were incubated in the presence of IFN-γ (300 U/ml), and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) Representative western blot of iNOS expression. Cells were incubated in the presence (+) or the absence (–) of IFN-γ and proteins were isolated at different times. β-actin was used as loading and transfer control. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). *, $p < 0.05$ (ANOVA and post-hoc Tukey's test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h in the presence of IFN-γ.

specific leukocytes.[7,10,31] The oligomerization of several of these subunits might contribute to the diversity of K^+ currents, but the participation of modulatory subunits must also be considered. Regulatory Kv subunits are primarily distributed into three main groups: (1) the Kvβ subunit gene family, with three major gene products, *Kvβ1*, *Kvβ2* and *Kvβ3*;[11] (2) the KCNE family, which comprises five different members (KCNE1–5);[14] and (3) the potassium channel-interacting (KChip) and potassium channel-accessory (KChap) proteins, which form an heterogeneous group of peptides.[32,33] While the last group is primarily considered as a collection of chaperones, which almost exclusively modulate the level of channel surface expression, the former groups also control kinetics. There is a considerable amount of data concerning the Kvβ interaction with Kv channels and the identification of these subunits in leukocytes. However, the presence and the regulation of KCNE subunits in the immune system have not been thoroughly addressed, although sound evidence indicates that KCNE peptides might modulate leukocytic Kv channels.[21]

The present work is the first to systematically analyze the expression of KCNE1–5 mRNA in differentiated leukocytes, such as macrophages and B and T lymphocytes. Although all leukocytes expressed KCNE1–5, the mRNA abundance varied among the cell lineage. Preliminary and heterogeneous affymetrix gene chip studies have previously addressed this issue leading to controversial interpretations as discussed below (see **Supplemental**

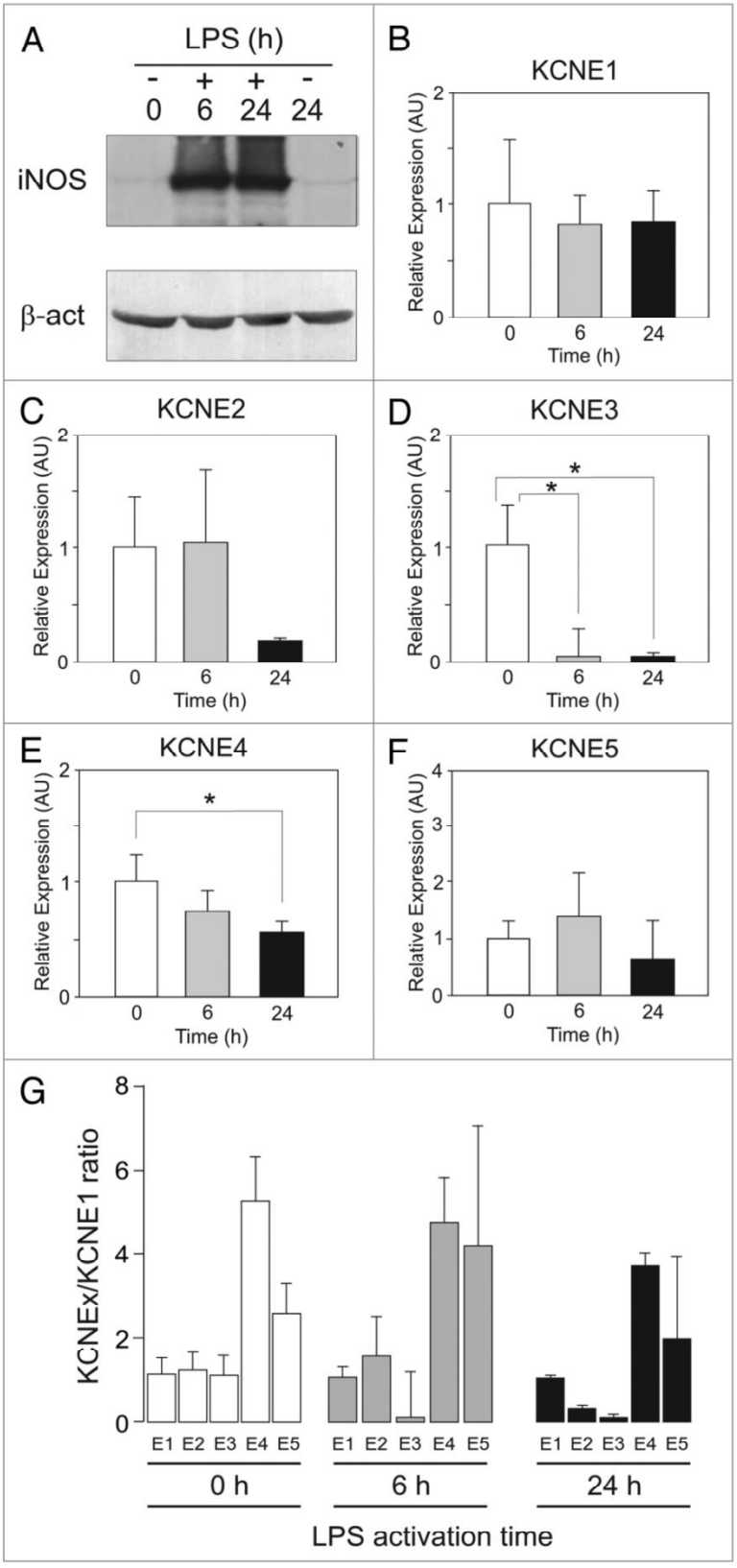

Figure 3. Expression and regulation of KCNE (1–5) mRNA in LPS-activated BMDMs. BMDMs were incubated in the presence of LPS (100 ng/ml), and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in Materials and Methods. (**A**) Representative western blot of iNOS expression. Cells were incubated in the presence (+) or the absence (–) of LPS and proteins were isolated at different times. β-actin was used as loading and transfer control. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. Values are the mean ± SEM (n = 3–6). *, $p < 0.05$ in KCNE3 (Student's t-test). *, $p < 0.05$ in KCNE4 (ANOVA and post-hoc Tukey's test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h in the presence of LPS.

our work relies on the KCNE gene expression and possible post-transcriptional modifications should be contemplated for future research.

Because Kv channels play crucial roles in the immune response, we further studied KCNE mRNA regulation upon leukocyte proliferation and activation. A few studies have documented the expression of KCNE subunits in the immune system, but show contradictory results. In 1992, Attali and coworkers isolated KCNE1 from Jurkat T cells, but reported no changes upon activation.[16] Chouabe et al. (1997) confirmed the expression of KCNE1 in peripheral blood leukocytes but observed no expression in the spleen and thymus.[34] The results of these pioneering studies have led to controversial debates concerning which cell lineage expresses KCNE1, and what state of differentiation/maturation/activation is required to detect KCNE1 expression. Here, we further confirmed that KCNE1 expression, which is scarcely detected, remained almost consistent upon proliferation or under various insults in macrophages and B and T lymphocytes. Our results are consistent with those of Lunquist et al. (2006) who demonstrated that the KCNE1 mRNA is the least abundant subunit in thymus, spleen and peripheral leukocytes.[35] However, the array data from different sources support contradictory results, arguing that there is similar mRNA expression among the KCNEs, with no changes in neoplastic growth between the lineages (GeneChips from GeneNote, http://bioinfo2.weizmann.ac.il/cgi-bin/genenote/home_page.pl and GNF BioGPS, http://biogps.gnf.org, see **Supplemental Material** for extended information). This could be characteristic of the immune system because the upregulation of KCNE1 has been previously shown under neoplastic growth in other tissues.[36,37] Similar differential tissue-specific responses have also been demonstrated with Kv1.3 and Kv1.5 in macrophages and muscle cells.[3,38]

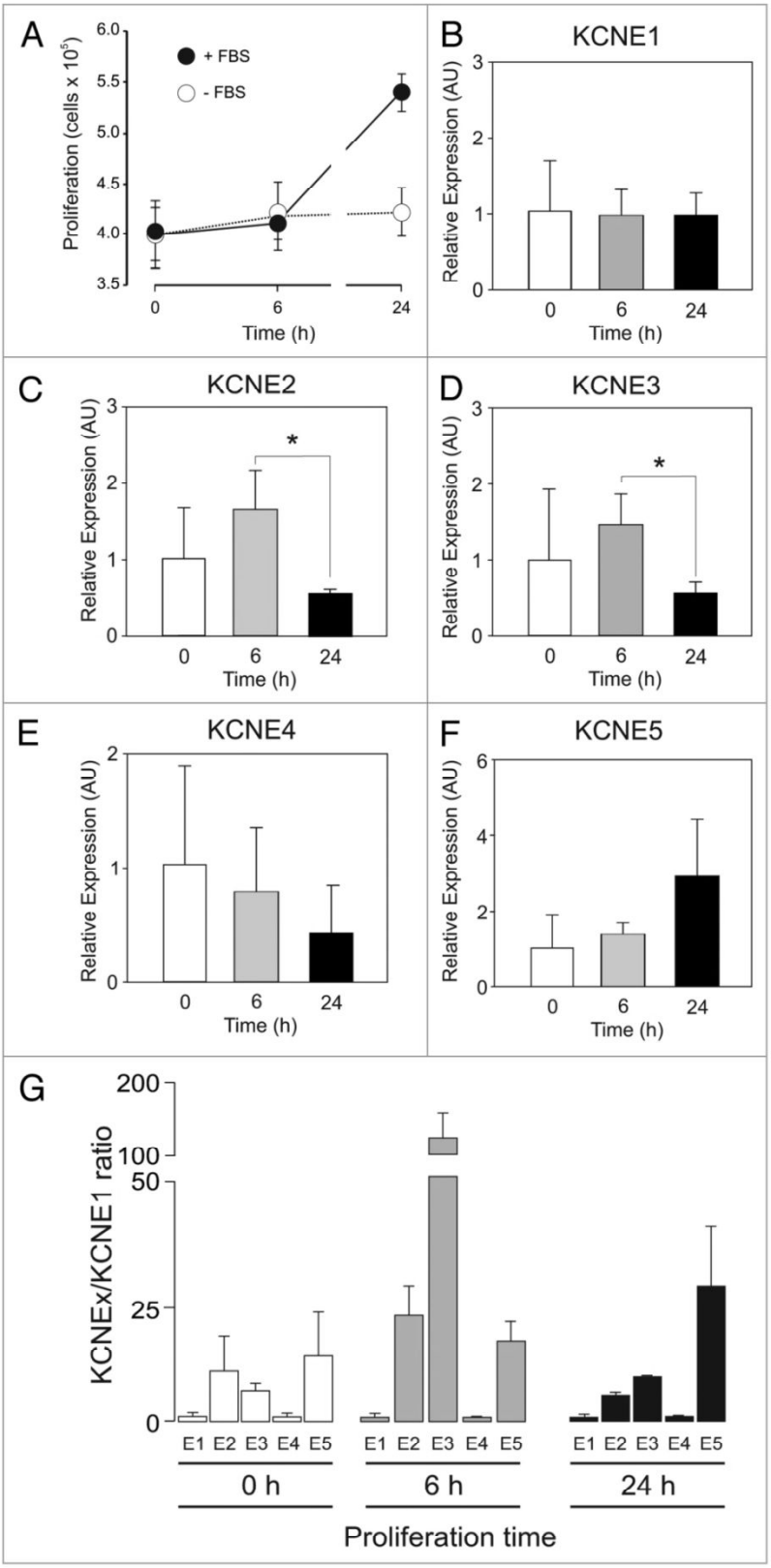

Figure 4. Expression and regulation of KCNE (1–5) mRNA in FBS-dependent Raji B-lymphocytes proliferation. G_0-arrested B-cells were incubated in the presence of FBS, and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) FBS-dependent proliferation of Raji B cells. The values are given as the mean ± SEM and represent cell number of four independent experiments each performed in triplicate. ●, + FBS; ○, - FBS. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). *, $p < 0.05$ (Student's t-test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h of FBS-dependent proliferation.

Are there other KCNEs expressed in the immune system? The expression of other KCNEs has not been thoroughly examined in leukocytes. The expression of KCNE2 in leukocytes and tissues from the immune system is limited.[35] We observed that MCSF and FBS induce a notable cell cycle-dependent upregulation of KCNE2 mRNA. However, affymetrix GeneChip studies show that KCNE2 mRNA abundance is similar between cancerous and healthy thymus, bone marrow and whole blood specimens. In contrast, activation triggers the downregulation of KCNEs in Jurkat T cells, but other insults failed in macrophages or Raji B cells. The results obtained from array data from experiments performed in different leukocytic, human leukemia and lymphoma cell lines suggest that KCNE2 expression is quite stable (see **Supplemental Material** for GeneChip information). Changes during the cell cycle and/or differentiation might explain these differences. In fact, in most cases, we report that the upregulation of KCNE2 was transient and returned to basal levels during late phases of the cell cycle.

The expression of KCNE3 presents an interesting debate. While Abbott et al. (2001) reported no expression of KCNE3 in thymus, spleen and human leukocytes[39] Lundquist et al. (2006) observed the abundant expression of KCNE3 in the same tissues.[35] Other evidence also indicates a disparity among cell lines. For instance, array data from GNF BioGPS has shown that KCNE3 might be prominent in cells of a myeloid origin (see **Supplemental Material** for GeneChip information). Our data do not support the results obtained from the last study because KCNE3 expression was low

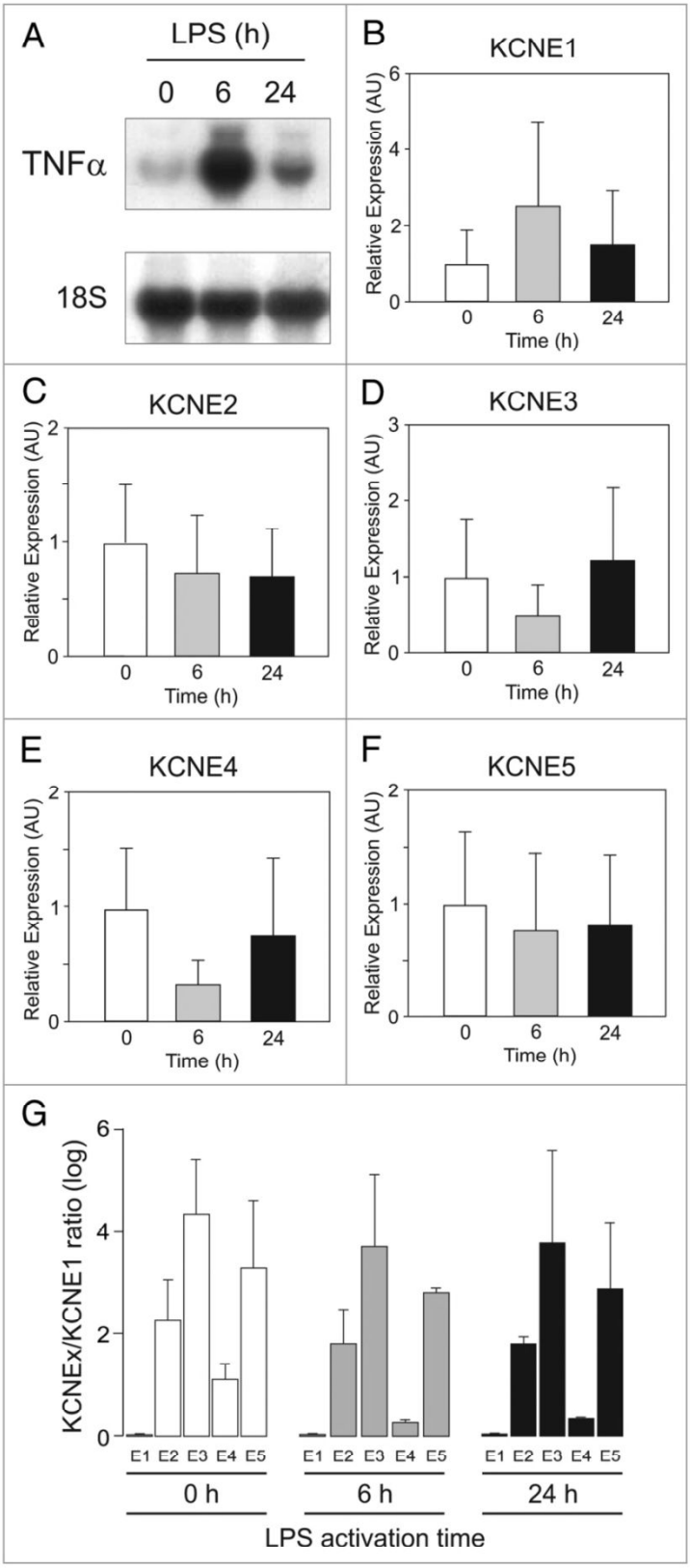

Figure 5. Expression and regulation of KCNE (1–5) mRNA in LPS-activated Raji B lymphocytes. B cells were incubated in the presence of LPS (100 μg/ml), and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) TNFα mRNA expression. Ribosomal 18S RNA was used as control. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h in the presence of LPS.

in macrophages. However, KCNE3 mRNA suffers differential regulation between proliferation and activation with a remarkable downregulation under LPS and IFN-γ incubation. Furthermore, we did observe notable KCNE3 mRNA expression in human B and T lymphocytes, with minor regulation upon proliferation or activation.

Grunnet et al. (2003) documented the consistent expression of KCNE4 in activated T lymphocytes.[17] KCNE4 expression is relatively abundant in the immune system (see supplemental material for GeneChip information). Although most studies show that KCNE4 mRNA levels are slightly higher than other KCNEs in the spleen, multiple native leukocytes and several leukemia and lymphoma cell lines show little expression in peripheral leukocytes.[35] Our data support the idea that the mRNA levels of KCNE4 are quite abundant, and experienced interesting regulation. We have previously reported that while KCNE4 expression increased in LPS-activated Raw 264.7 macrophages, the immunosuppressant dexamethasone triggered no changes.[20] In the present work, we further documented the cell cycle-dependent upregulation of KCNE4 in BMDM, whereas the levels of mRNA remained constant or decreased upon IFN-γ and LPS incubation, respectively. Unlike in Raw cells, the presence of LPS and IFN-γ inhibits proliferation in BMDMs.[22,23] IFN-γ and LPS arrests cells at the G1/S boundary, but only LPS induces apoptosis.[23,24] Thus, Kv1.3, a documented target of KCNE4-dependent negative regulation, potentially plays a dual role in proliferation and apoptosis.[40] Therefore, it is tempting to speculate that the Kv1.3 activity should be conserved during proliferation and apoptosis in BMDMs. We observed similar regulation in T lymphocytes but not in B cells, where KCNE4 mRNA levels remained consistent.

Finally, KCNE5 is the less documented KCNE.[35] The results of affymetrix GeneChip studies (see **Supplemental Material** for GeneChip information) have demonstrated that KCNE5 is present in cells and healthy and cancerous tissues from the immune system. However, no KCNE5 regulation has been documented. We observed the cell cycle-dependent regulation of KCNE5 in BMDMs and a steady increase in activated T-lymphocytes. In general, our results would support quite notable levels of KCNE5

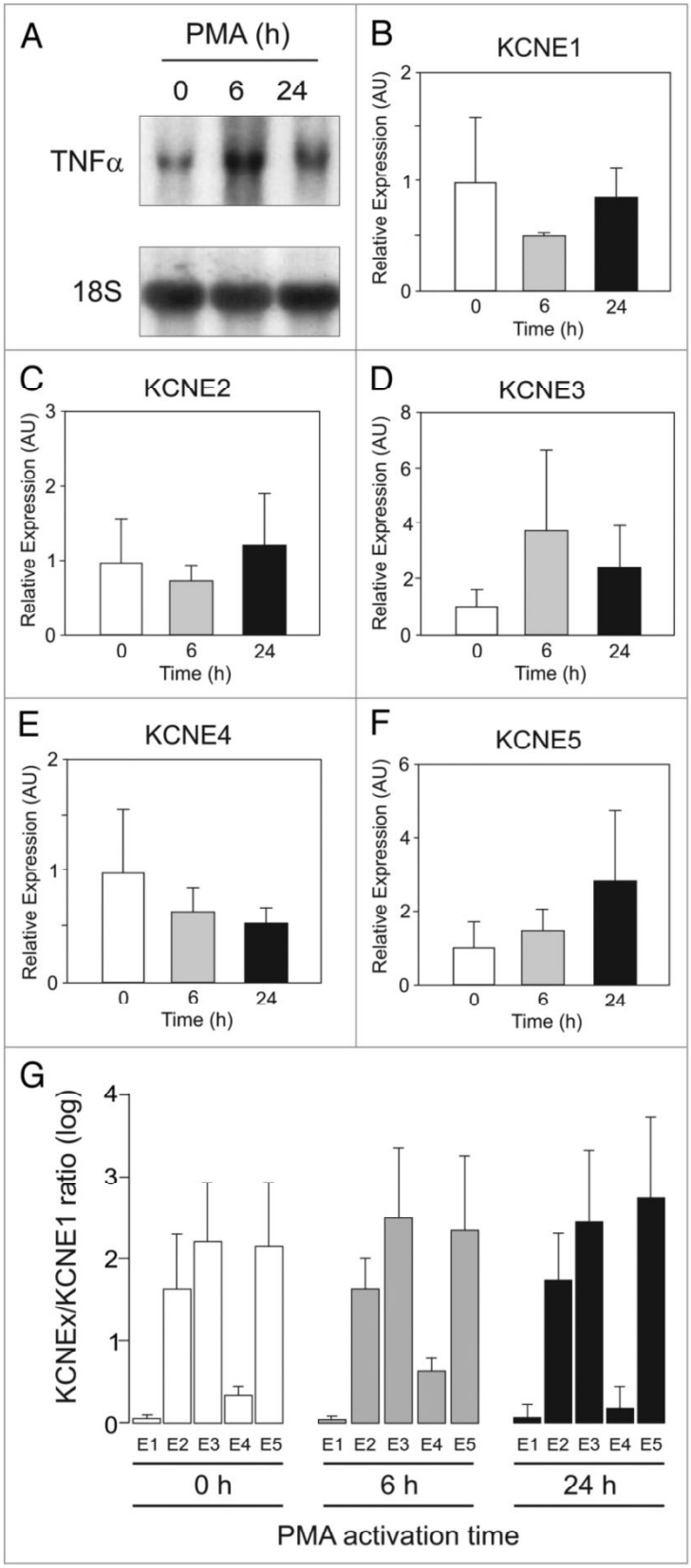

Figure 6. Expression and regulation of KCNE (1–5) mRNA in PMA-activated Raji B lymphocytes. B cells were incubated in the presence of 10 nM PMA and mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) TNFα mRNA expression. Ribosomal 18S RNA was used as control. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h in the presence of PMA.

mRNA in leukocytes consistent with the highest levels detected in thymus and myeloid cells (see **Supplemental Material** for GeneChip information).[35]

In summary, our work systematically addresses, for the first time, the mRNA expression of the five KCNE subunits in leukocytes. These newly identified auxiliary leukocytic channel subunits might exert regulatory actions, such as the regulation of Kv gating, traffic and localization. This field is in its infancy and these data serve as a useful roadmap to inform future studies and represents a step toward understanding the role of these regulatory subunits in leukocyte physiology.

Materials and Methods

Animals, cell culture and proliferation assays. Murine bone marrow-derived macrophages (BMDM) from 6- to 10-wk-old BALB/c (Charles River laboratories) were used. The cells were isolated and cultured as described elsewhere.[3,22] Briefly, animals were sacrificed by cervical dislocation, and the adherent tissue was removed to dissect both femurs. The ends of bones were cut off, and the marrow tissue was flushed using irrigation with medium. The marrow plugs were passed through a 25-gauge needle for dispersion. The cells were cultured in DMEM containing 20% FBS and 30% L-929 fibroblast (L-cell) conditioned media as a source of macrophage colony-stimulating factor (MCSF). The macrophages were obtained as a homogeneous population of adherent cells after 7 d of culture and maintained at 37°C in a humidified incubator under 5% CO_2. For the experiments, the cells were cultured with tissue culture differentiation medium (DMEM, 20% FBS, 30% L-cell medium) or arrested at G_0 using MCSF deprivation in DMEM supplemented with 10% FBS for at least 18 h. The G_0-arrested cells were further incubated in the absence or the presence of recombinant murine MCSF (1200 U/ml), with or without lipopolysaccharide (LPS, 100 ng/ml, Sigma-Aldrich) and with or without IFN-γ (300 U/ml, Preprotech). The ethics committee from the University of Barcelona approved all methods for animal care and handling, and the experiments were conducted in accordance with EU regulations.

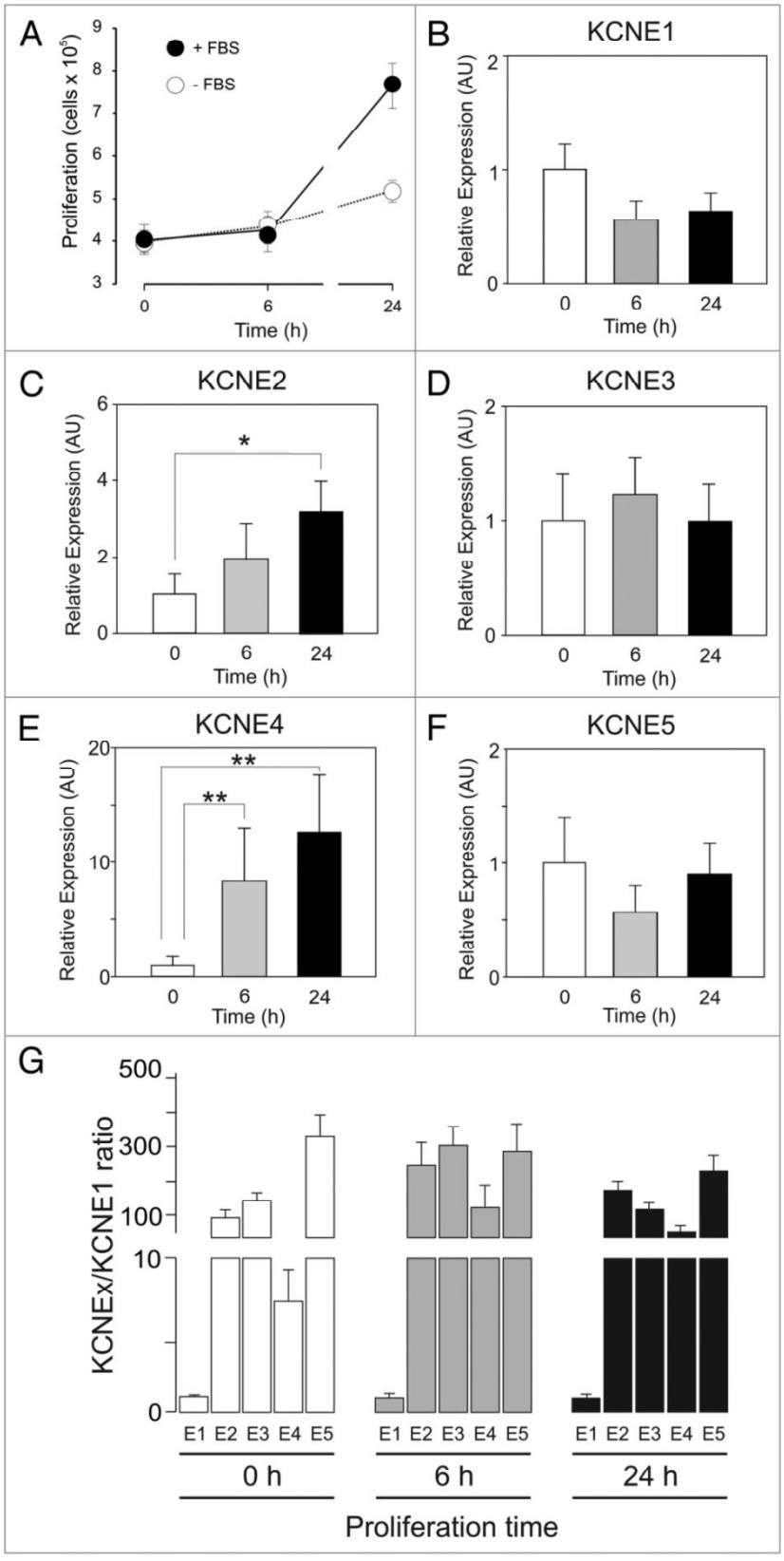

Figure 7. Expression and regulation of KCNE (1–5) mRNA in FBS-dependent Jurkat T-lymphocyte proliferation. G_0-arrested T cells were incubated in the presence of FBS, and the mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) FBS-dependent proliferation of Jurkat T-cells. The values are given as the mean ± SEM and represent cell number of four independent experiments each performed in triplicate. ●, + FBS; ○, - FBS. (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). *, $p < 0.05$; **, $p < 0.01$ (ANOVA and post-hoc Tukey's test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h of FBS-dependent proliferation.

Raji human B lymphocytes and Jurkat human T lymphocytes were routinely cultured in RPMI culture media containing 10% FBS and supplemented with 10 U/mL penicillin and streptomycin. In proliferation experiments, the Raji B cells and Jurkat T cells were incubated for 24 h in RPMI media without FBS supplemented with 0.02% BSA (G_0-arrested cells) prior to culture in regular RPMI supplemented with 10% FBS. While 10 nM phorbol ester (PMA, Sigma-Aldrich) and LPS (100 μg/ml) were used to activate B lymphocytes, the Jurkat T cells were stimulated with 5 μg/ml of phytohematogglutinin (PHA, Sigma-Aldrich) supplemented with 80 nM of PMA. Control cells treated with DMSO, the vehicle for PMA, were used to discard possible non-specific side effects.

Proliferation was investigated by analyzing the cell number and viability. A Countess™ automated cell counter (Invitrogen) was used for cell counting and viability analyzed by means of tripan blue exclusion. Viabilities higher than 85 ± 15% were used in all groups.

RNA isolation, RT-PCR analysis and real-time PCR. Cells cultured in 100-mm tissue culture dishes were incubated for a 24 h test period before harvesting, with agents added 6 or 24 h before harvesting or with no agents added ("0 h," see **Fig. S2**). Total RNA from BMDM, Raji B cells and Jurkat T cells was isolated using the Nucleospin RNA II kit (Invitrogen), which contains DNase I. PCR controls were performed in the absence of reverse transcriptase. cDNA synthesis was performed using Transcriptor Reverse Transcriptase (Roche) with a random hexanucleotide and oligo(dT)

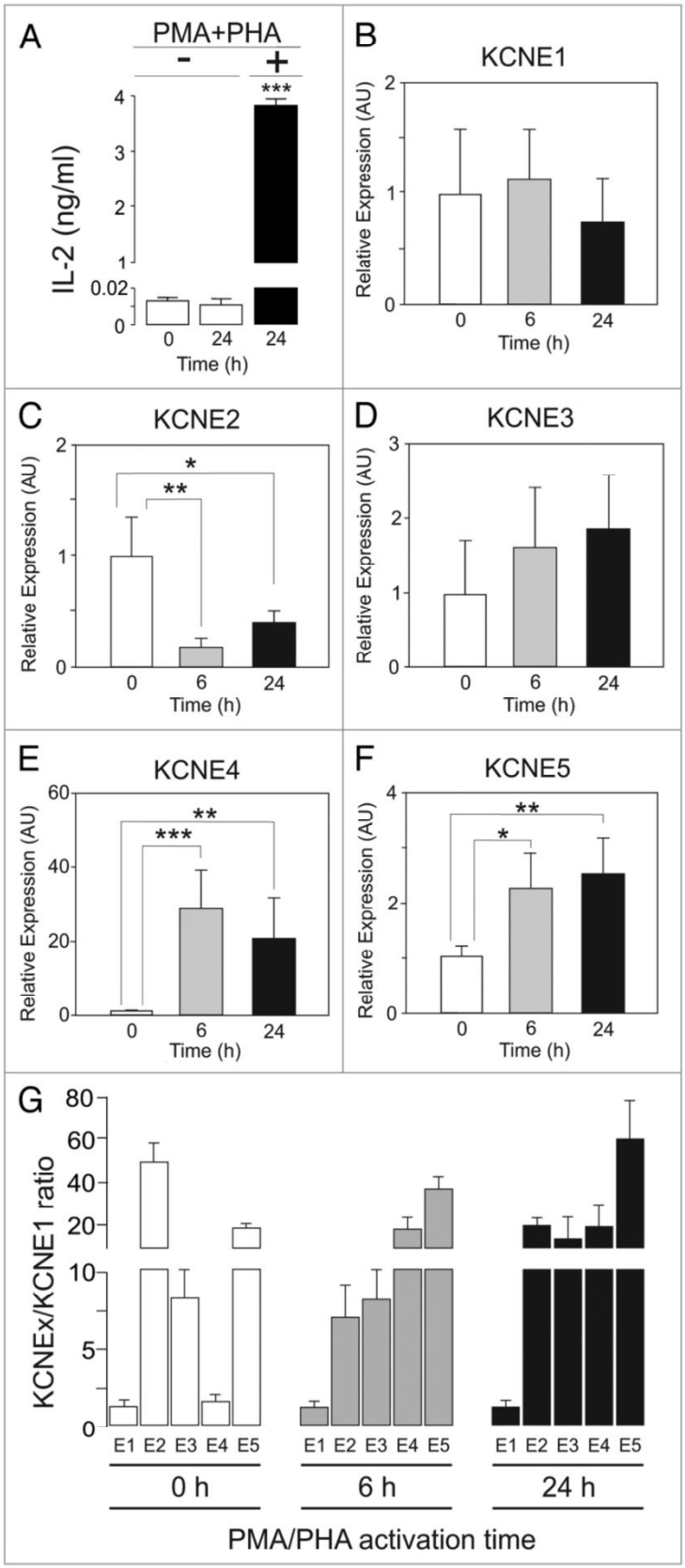

Figure 8. Expression and regulation of KCNE (1–5) mRNA in PMA/PHA-activated Jurkat T lymphocytes. T cells were incubated in the presence of PMA/PHA (80 nM/5 μg/ml), and mRNA was isolated simultaneously after the indicated times of treatment. Real-time PCR was performed as described in *Materials and Methods*. (**A**) IL-2 production (ng/ml) of control (-) and PMA/PHA-stimulated Jurkat T lymphocytes (+). ***, $p < 0.001$ vs. non treated (-). Open bars, absence of PMA/PHA; closed bar, presence of PMA/PHA. Values are mean ± SEM (n = 4). (**B–F**) Relative mRNA expression, in arbitrary units (AU), of KCNE1–5. (**B**) KCNE1; (**C**) KCNE2; (**D**) KCNE3; (**E**) KCNE4; (**F**) KCNE5. The values are given as the mean ± SEM (n = 3–6). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test). (**G**) KCNEx/KCNE1 ratio. The relative expression of each KCNE and the KCNEx/KCNE1 ratio was calculated using the KCNE specific real-time PCR efficiency. See *Materials and Methods* for details. White bars, 0 h; gray bars, 6 h; black bars, 24 h in the presence of PMA/PHA.

primers according to the manufacturer's instructions. Real-time PCR was performed using a thermocycler (Applied Biosystems) and the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) according to the manufacturer's instructions. The PCR primer sequences, gene accession numbers, annealing conditions and amplicon lengths for each KCNE are listed in **Table 1**. The reactions were performed under the following conditions: an initial incubation at 95°C for 10 min followed by cycling at 95°C for 5 sec, specific annealing at T°C for 8 sec, and 72°C for 6–9 sec, and a final incubation at 95°C for 10 min. Melting curves were performed to verify the specificity of the product, and ribosomal 18S RNA was included as an internal reference as previously described.[3,12] The results were analyzed using commercial software (Applied Biosystems). For each primer set, a standard curve was generated, and the slope factor was calculated. The values were normalized to the corresponding 18S RNA. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation $E = 10^{(-1/slope)}$. The normalized KCNEx/KCNE1 ratio was calculated taking into account the primer pair efficiencies as follows: Ratio = $(1+E)^{\Delta Ct\ (KCNEx)} / (1+E)^{\Delta Ct\ (KCNE1)}$, where Ct signifies the threshold cycle.[41]

Northern blot analysis. Up to 20 μg of total RNA was fractionated by electrophoresis through a 1% agarose, 3% formaldehyde gel in 20 mm MOPS and 1 mm EDTA, pH 7.4. Application of equal amounts of RNA to each lane was confirmed by the addition of ethidium bromide to the samples before electrophoresis. RNA was transferred overnight to an Immobilon filter (Amersham Pharmacia Biotech) by capillary action in 20 × SSC (SSC, 3 min NaCl, 300 mm sodium citrate, pH 7.0). RNA was cross-linked to the filter by irradiation with UV light. Filters were hybridized with hTNF-α and rat 18 S rRNA cDNA probes. Filters were washed once for 30 min at 65°C in 3 × SSC and 1% lauryl sulfate, once in 1 × SSC and 1% lauryl sulfate, and once with 0.2 × SSC and 1% lauryl sulfate before autoradiography.

Table 1. PCR primers, accession numbers, annealing conditions and amplicon lengths for each KCNE

	Sequence	Accession number	Annealing (°C)	Amplicon (bp)
Mouse KCNE1	F: 5'-TGA CAG CAA GCA GGA GGC GCT-3' R: 5'-GAC ATA GCA AGC TCT GAA GC-3'	NM_008424	58	210
Mouse KCNE2	F: 5'-GTC ATC CTG TAC CTC ATG GT-3' R: 5'-TTC TCA TGG ATG GTG GCC TT-3'	NM_134110	60	194
Mouse KCNE3	F: 5'-CGT AAT GAC AAC TCC TAC ATG-3' R: 5'-TAC ACA TGA TAG GGG TCA CT-3'	NM_020574	67	127
Mouse KCNE4	F: 5'- TCC AGC AGC CCC CTC GAG TC-3' R: 5'-AGG CTG GAC TTC TTC TCC CG-3'	NM_021342	60	158
Mouse KCNE5	F: 5'-ACC CCT ACC CCG CAT ATC CAA-3' R: 5'-TTG GAC GTG CTG GAT TCT GTT-3'	NM_021487	55	106
Human KCNE1	F: 5'-TGT GCC TGG GAA GTT TGA G-3' R: 5'-GGG AAC CAC TGA CAG TCT ATC-3'	NM_000219	70	634
Human KCNE2	F: 5'-ACG CCA GCA AGA AGG TTC A-3' R: 5'-CTT GCT CAG CTG TTG TGT TC-3'	NM_172201	60	183
Human KCNE3	F: 5'-GCT AAT ACA TGC AAT GTG GC-3' R: 5'-TTC AAT GGG TAC TTC CAG G-3'	NM_005472	55	137
Human KCNE4*	Quantitect primer assay from Qiagen Ref QT00046676	NM_080671	55	105
Human KCNE5	F: 5'-CCT ACC CCG CAC ATC CAA CTG CAC-3' R: 5'-TTG GAC GTG TTG GAT TCA GTT CC-3'	NM_012282	62	104
18S	F: 5'-CGC AGA ATT CCC ACT CCC GAC CC-3' R: 5'-CCC AAG ATC CAA CTA CGA GC-3'	NR_003286 Human NR_003278 Mouse	55	210

F, forward; R, reverse. *For hKCNE4 a validated Quantitect primer assay from Qiagen (Ref QT00046676) was used with no available primer sequences.

Western blot analysis. Cell extracts for Western blot analysis were obtained after washing the cells twice in cold phosphate-buffered saline followed by lysis in 1% Nonidet P-40, 10% glycerol, 50 mm HEPES, pH 7.5, and 150 mm NaCl, supplemented with a protease inhibitor mixture consisting of 1 μg/ml aprotinin, 1 μg/ml leupeptin, 86 μg/ml iodoacetamide, and 1 mm phenylmethylsulfonyl fluoride. Protein was determined following the Bradford technique (Bio-Rad). Protein samples (20 μg) were incubated for 6 min at 95°C prior to 10% SDS-PAGE. Proteins were transferred onto filters (Immobilon-P, Millipore), which were then blocked in a 5% dry milk-supplemented 0.2% Tween 20, phosphate-buffered saline prior to immunoreaction. To monitor the expression of iNOS, a commercial rabbit antibody against mouse iNOS (M-19, Santa Cruz Biotechnology) was used. As a loading and transfer control for this technique, a mouse anti-mouse β-actin antibody (Sigma) was used.

IL-2 production measurements. IL-2 production in Jurkat T-lymphocytes was measured with an ELISA kit (eBioscience), following the manufacturer's instructions. Cells were centrifuged at 1200 rpm for 4 min, and the supernatant was used to quantify the IL-2 concentration. Reactions were performed in 96-well plates coated with the capture antibody. After blocking, standards and samples were incubated with Detection Antibody followed by HRP-Streptavidin. H_3PO_4 (1M) was used as stop solution. Plates were read at 450 nm.

Statistics. The values are expressed as the mean ± SEM of 3–6 experiments performed in triplicate. The significance of the differences was established using Student's t-test or one-way ANOVA followed by a post-hoc Tukey's test (GraphPad, PRISM 5.0) where indicated. The decision to perform one-way ANOVA analysis was considered when significance was not observed using Student's t test. A value of $p < 0.05$ was considered significant. Due to the mathematical transformation, the statistical significance of the normalized KCNEx/KCNE1 ratio was considered irrelevant. The data should be considered qualitatively illustrative rather than a quantitative value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Supported by the Ministerio de Economia y Competitividad (MINECO), Spain (BFU2011-23268 and CSD2008-00005 to A.F., BFU2010–15674 to C.S.). L.S., A.V.G., S.R.R. and D.G. hold fellowships from the MINECO. J.M. was an APIF fellow (Universitat de Barcelona). L.S. and A.V.G. contributed equally. C.S. and A.F. contributed equally.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/chan/article/23258

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7. ANNEX

Volts de mitjanit

Albert Vallejo Gracia

Dissabte als volts de mitjanit. És fosc. Fa fred i el cel és ple de núvols. Sembla que hi haurà tempesta, una d'aquestes tempestes elèctriques. Tot i que la resta del grup no ha volgut animar-se a prendre alguna cosa, en Goldman i el seu company solter d'aventures, en Nersnt, sí que han decidit sortir una estona. Els dos col·legues són a dins del local, arrecerats de la intempèrie, xerrant en una taula que hi ha sota una de les múltiples finestres, amb unes copes d'aquelles que treuen el fred de cop i que apunten a una gran nit. La sala està encara una mica buida i la freqüència musical té un ritme prou calmat. Això els permet comentar els darrers esdeveniments esportius i riure també, una estona, dels malanomenats salaos dels seu amics, per preferir quedar-se a casa amb les seves parelles enlloc de ser aquí amb ells. El tema és prou suculent, com per a que decideixen demanar una altra ronda per tal de solvatar-se una mica més.

Van passant les hores i l'ambient continua massa calmat, com en un estat estacionari. En Nersnt comença a neguitejar-se, perquè veu que, tot i el transcurs de les hores, tot continua molt tranquil. Sent que o bé s'anima o bé entrarà a un pou de cansament. De fet, li pregunta al seu col·lega, "Qui has dit que punxava aquesta nit?" En Goldman, que nota que el seu amic comença a estar una mica perjudicat, respon que creu que aquesta nit serà la DJ TTX Sensitive current qui tocaria. El company replica que tant de bo valgui la pena i que sigui aviat, que ja és tard i no es troba gaire bé. Diu sentir-se fora de lloc i de l'equilibri, i que té força ganes de marxar a casa i estar en repòs. Però en Goldman sap que el seu amic s'agobia, sobretot, per l'absència d'afluència d'elements femenins diferents als que ja està acostumat a veure dia sí, dia també. Esperava que avui dissabte hi hagués una amalgama variada i diversa.

Per sort, en qüestió d'uns breus moments, l'artista convidada enceta un concert amb uns ritmes que delectaran al públic. Amb el seu repertori provoca que el microambient del local canviï radicalment i lenta. Els joves de la zona comencen a entrar al que ara sembla cada cop més una discoteca. Poc a poc, va formant-se cua a l'entrada. El mal temps, sumat a les bones vibracions que emanen del local, fan que totes les espècies vulguin entrar a dins. La capacitància del local ha arribat al seu límit, comença a no poder-hi entrar ningú més. En Goldman i el Nernst continuen gaudint de la música des del seu lloc privilegiat. En Nernst, ara molt més animat i amb un gran potencial, no para de mirar les corrents de gent que entren a batzef. "Se't gastaran els ulls de tan mirar-les, Nernst!" li diu amb aire fatxenda en Goldman al seu company de nits memorables que no para de repassar a totes i cadascuna de les benvingudes. "Tio! És que mira com vesteixen! Realment fan honor al seu nom!" respon en Nernst tot rient. En Goldman, sobtat pel comentari, replica al seu col·lega que no es passi, i no entén com pot congeniar amb un paio tan masclista. Li recomana passar una mica desapercebut, ja que el goril·la de l'entrada no para de fer fora a habituals del local. Es fixen, i realment surten tres nois per cada dues noies que entren. Més els val, doncs, passar ben desapercebuts.

La DJ és realment extraordinària. De mica en mica, pols a pols, aconsegueix embriagar a la gent. L'atmosfera esdevé cada cop més positiva. La música cada cop sona més forta, més intensa. Tothom xerra, tothom beu. Els cambrers van tal atrafegats, que semblen missatgers que vagin de banda a banda del bar, tot esquivant els grups de gent que de mica en mica van acumulant-se dins. El càlid ambient es torna cada cop més despolaritzant i desinhibit, i això contrasta amb la violenta i freda tempesta de l'exterior. En Goldman balla com si no hi hagués demà, mentre que en Nernst diverteix a un grup de noies amb somriures d'amplitud considerable. De forma màgica, música i públic creen una sinèrgia espectacular. El ball i el moviment dels uns, inspiren d'una forma elèctrica allò que sona, i modulen el voltatge com un remolí ascendent. Es respira una energia tal, que sembla que cap llei imperi en aquest microdomini. Es capta tal potencial, que els desigs entròpics aparenten dominar el present. Ni forces químiques, ni elèctriques. Allò que sembla

regir i imperar en aquesta petita festa és un gradient de gresca i llibertat. La música ho envolta tot i el voltatge no para de pujar, in crescendo, superant qualsevol sostre o llindar. Volt a volt. Poc a poc. Tots els cors bateguen. Tots els àtoms vibren. I és aleshores quan, també i com tot, en qüestió de mil·lisegons, se sent un fort soroll de trencadís.

Per uns breus instants es fa el silenci i la música s'atura. Lentament se sent un fred gèlid i el soroll de trons i llampecs. I aleshores, de cop i volta, uns crits de dolor i pànic se senten aquí i allà. El voltatge i el ritme frenètic dels convidats havien provocat que, estocàsticament, s'esquerdessin i es trenquessin els vidres de les finestres. Aquests han ferit greument a diverses persones. Tot això passa de forma tan sobtada que, tant als dos amics com a la resta de presents, els costa adonar-se del fet que fa uns instants estiguessin ballant al ritme d'una àlgida excitació i que, ara, el fred i la intensa pluja de l'exterior els envaïssin malèvolament. La momentània passivitat marxa tan fugaç com ha vingut i el caos torna a governar. L'aigua i el vent entren a bots i barrals, de forma violenta i a contracor del públic assistent, gairebé per osmosi inversa. El pànic engendra crits, i la cridòria fluxos per sortir del bar. La porta principal està tan col·lapsada que les desesperades noies s'amunteguen al voltant de les petites finestres per tal d'escapar i fugir d'aquest infern, a favor de gradient i en cerca del descans i del seu repòs. S'empenyen les unes contra les altres. La pretèrita festa serà ara un record de nostàlgia al nucli de tots i cadascun dels assistents. En Nernst i en Goldman, espantats, molls de cap a peus i amb alguna que altra ferida, es lamenten del fatal esdevenir de la nit. Sabien que estava essent una d'aquelles nits memorables i d'alt voltatge. Donat que ambdós són, com la resta de nois del local, de forma significativa, massa voluminosos per a sortir a través d'aquelles finestres, resten a la darreria tot comentant l'estrany comportament de les finestres i la seva dèbil resistència. Algú comenta que aquest tipus de finestres de baix cost i aquesta falta de control i de seguretat només les havien vist anteriorment en discoteques poc concorregudes i perilloses. En Nernst diu conèixer-les i recorda alguns noms de locals com Bar Autoimmunitat o Discoteca Neoplàsia. Veient que algú està ajudant als cambrers en la tasca d'anar tancant i segellant una a una totes les finestres amb cordes i fustes, els dos amics decideixen donar un cop de mà per tal de reestablir de nou l'orde i l'estat de repòs.

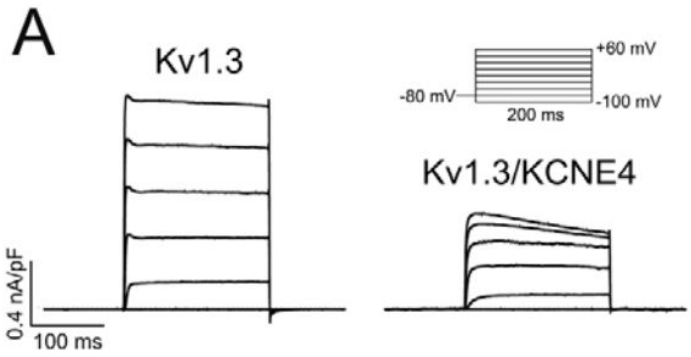

Figura 1. El KCNE4 modifica l'apertura del K_v1.3. *Registres electrofisiològics representatius obtinguts de cèl·lules HEK293 expressant Kv1.3 sol i Kv1.3/KCNE4. O bé, les finestres obertes i tancades. (Solé et al., 2009).*

Finalment, i ja de ben entrada la matinada, els cambrers pregunten a en Goldman i a en Nernst si volen que els convidin a una última copa, per les molèsties causades. "Na+, ja n'hem tingut prou per avui!" responen alhora els dos ions de sodi.

Albert Vallejo Gracia. Volts de mitjanit. Biologia On Line (revista de Divulgació de la Facultat de Biologia). 2015. Jul, 4, 2. *Ier premi del I Concurs de Microrelats de Ciència en Ficció de la Facultat de Biologia, Abril 2015.*