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Research Article

Selective Inhibition of Intracellular Kv1.3 Potassium Channels by Lentivirus-Mediated Expression of Agitoxin

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pLL3.7 MCS
Nhe-Xho-ER1-Xba-Not

Oligonucleotides for oligo-anneal:
Agitoxin

Kozak M G V P I N V S C T G S P Q C I K P C K D A
Top 5’ ctagc gccacc atg ggc gtg cct atc aac gtt tcc tgt acc ggc tcc cct caa tgt atc aaa cct tgt aaa gac gcc

G M R F G K C M N R K C H C T P K stop Not
ggc atg agg ttc ggc aaa tgc atg aat aga aag tgc cac tgt aca cct aag tga gc 3’

Bottom 5’ ggccgc tca ctt agg tgt aca gtg gca ctt tct att cat gca ttt gcc gaa cct cat cgg ggc gtc ttt aca agg ttt
gat aca ttg agg gga gcc ggt aca gga aac gtt gat agg cac gcc cat ggtggc g

Scramble

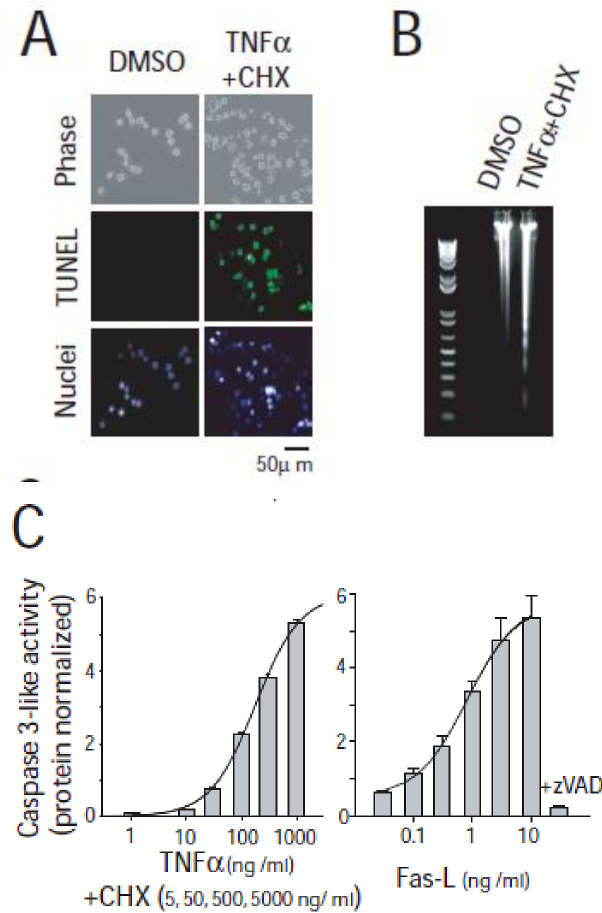
Kozak M I N D V A G R K C N Q S P C T K S P G P
Top 5’ ctagc gccacc atg atc aac gac gtg gcc ggc aga aaa tgt aat caa tcc cct tgc aca aag tcc cct ggc cct

T C M G P C I C M F K R K H K C V G stop
aca tgt atg ggc cct tgt atc tgt atg ttc aaa aga aaa cac aaa tgt gtg ggc tga gc 3’

Bottom 5’ ggccgc tca gcc cac aca ttt gtg ttt tct ttt gaa cat aca gat aca agg gcc cat aca tgt agg gcc agg gga
ctt tgt gca agg gga ttg att aca ttt tct gcc ggc cac gtc gtt gat cat ggt ggc g 3’

PCR oligonucleotides:		Nucleotides	NCBI ID
AgTX-F	5’ atggg cgtgc ctatc aacgt ttcc		synthetic
AgTX-R	5’ tcact taggt gtaca gtggc actt		
Scramble-F	5’ atgat caacg acgtg gccgg caga		synthetic
Scramble-R	5’ tcagc ccaca cattt gtgtt ttctt ttga		
EGFP-F	5’ atggt gagca agggc gagga gctgttc	n67-94	LC008492
EGFP-R	5’ gaagt cgatg ccctt cagct cgatgc	n435-460	
GAPDH-F	5’ ttcac cacca tggag aaggc t	n355-376	BC_083511
GAPDH-R	5’ ccctg ttgct gtagc caaat tcg	n1000-1023	
Kv1.3-FL-F1	5’ *gccacc* atgga cgagc gcctc agcct tctgc gctcg	n132-162	NM_002232
Kv1.3-FL-R1	5’ *acgcgt* aacat cggtg aatac ctttt tgatg tt	n1830-1857	
Kv1.3-FL-F2	5’ cgcga cttcc agcgc caggt gtgg	n772-796	
Kv1.3-FL-R2	5’ tcttc ccctt ctgtc tcccg gtgg	n1591-1615	
Kv1.3-Core-F	5’ *gccacc* atgag ctccg ggccg gcccg gggca tc	n815-839	
Kv1.3-Core-R	5’ *acgcgt* attga agttg gaaac aatca cggg	n1560-1583	

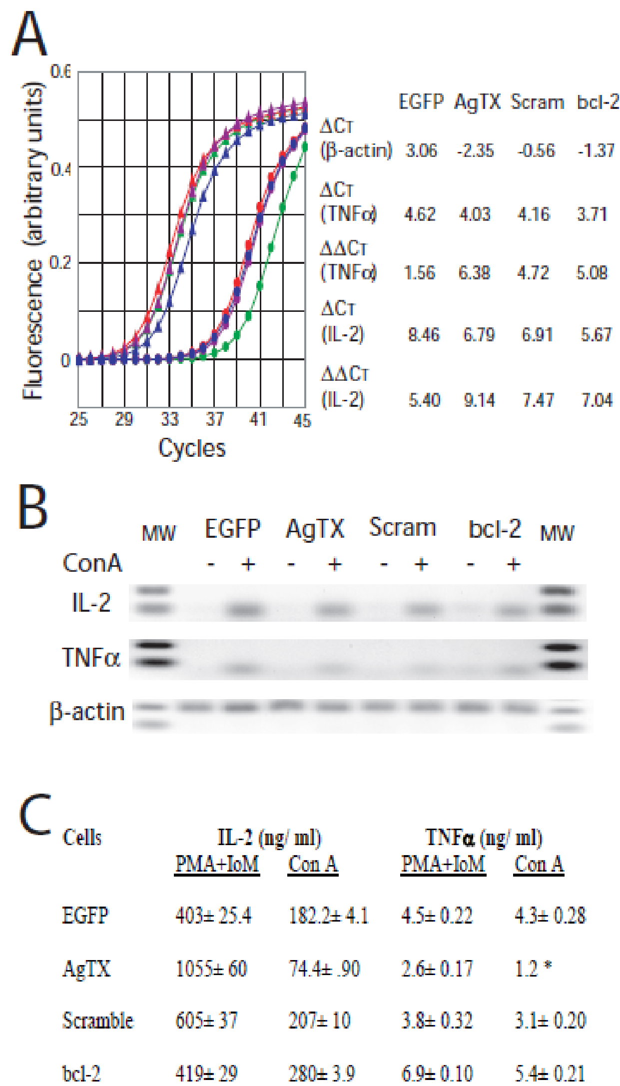
Supplemental Figure 1: Oligonucleotides used for creating the AgTX, scramble-AgTX, and Kv1.3 inserts. Two single stranded DNA oligonucleotides encoding the AgTX or scramble-AgTX representing the top and bottom strands were commercially synthesized (Integrated DNA Technology, Coralville, IA, U.S.A.), annealed, and kinased to create the phosphorylated double stranded inserts. The annealed inserts were designed to contain the sticky ends (underlined) for easy subcloning into the Nhe I and Not I restriction enzyme sites present in the multiple cloning site of the pLL 3.7 lentiviral vector.



Supp. Figure 2

Supplemental Figure 2: Induction of apoptosis in Jurkat cells by the sepsis-mimetic cytokine stimulation.

A. TUNEL staining of the cells after 3 hrs incubation with TNFα (100 ng/ ml) + cycloheximide (CHX) (500 ng/ ml). Control cells (DMSO) were incubated in the same amount of DMSO (0.1%). Approximately 35 % of cells were TUNEL (middle) positive and these cells showed condensed nuclei (bottom). The left panel is a phase contrast view of the same field. B. A 2% agarose gel image demonstrating DNA fragmentation by the same incubation with TNFα + CHX. C. Concentration-dependent induction of caspase 3-like activity (mean ± S.E.M.) by the same TNFα + CHX (left) or the Fas ligand (Fas-L) stimuli (right). The lines are concentration-response curve fit to the data with EC50 = 176.59± 28.32 ng/ ml, Hill coefficient = 1.06± 0.14 (for TNFα) and EC50 = 0.8± 0.08 ng/ ml, Hill coefficient = 1.02± 0.12 (for Fas-L). Note the complete inhibition of caspase activities by 1 μM zVAD.



	EGFP	AgTX	Scram	bcl-2
ΔCT (β-actin)	3.06	-2.35	-0.56	-1.37
ΔCT (TNFα)	4.62	4.03	4.16	3.71
ΔΔCT (TNFα)	1.56	6.38	4.72	5.08
ΔCT (IL-2)	8.46	6.79	6.91	5.67
ΔΔCT (IL-2)	5.40	9.14	7.47	7.04

Cells	IL-2 (ng/ ml)		TNFα (ng/ ml)	
	PMA+IoM	Con A	PMA+IoM	Con A
EGFP	403± 25.4	182.2± 4.1	4.5± 0.22	4.3± 0.28
AgTX	1055± 60	74.4± .90	2.6± 0.17	1.2 *
Scramble	605± 37	207± 10	3.8± 0.32	3.1± 0.20
bcl-2	419± 29	280± 3.9	6.9± 0.10	5.4± 0.21

Supplemental Figure 3: Transcriptional activation and release of the IL-2 and TNFα are preserved in Jurkat-AgTX cells. A. The denoted Jurkat cell lines were activated by Con A and mRNA harvested 24 hours later. The relative amounts of IL-2, TNFα, and β-actin mRNA were assayed by qRT-PCR. The amplification plots (left) were from Jurkat-EGFP (green), -AgTX (red), -scramble AgTX (purple) or -Bcl-2 (blue) mRNA templates at before (circle) and after (triangle) Con A treatment. The table (right) shows the normalized (ΔΔCt) mean values from a triplicate measurement and the experiment was repeated 3 times with similar results. A unit positive shift in ΔΔCt value (untreated - treated) indicates a theoretical maximum of 2-fold increase in the mRNA with treatment. B. RT-PCR products for IL-2 and TNF-α amplifications confirming the correct product size and the relative amounts. The amplification cycles were stopped near half-maximum to emphasize the difference in the product amounts but the same cycle number was used for the control and experimental amplification pairs for each cell lines. C. Proinflammatory cytokines released by activated Jurkat cell lines. The cell culture medium 24 hours after stimulation with the phorbol ester + ionomycin (PMA+IoM) or concanavalin A (Con A) were assayed for IL-2 and TNFα by ELISA. The pre-stimulation levels of the cytokines for all cell lines were below detection threshold (< 2.3

for IL-2 and <1.0 for TNFα). The numbers are mean ± S.E.M. from 6 measurements from 2 different experiments. * denotes measurements below the lower limit of detection.

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