Polymorphisms in *P2X4R* and *CAMKK2* may affect TNFα production: implications for a role in HIV-associated sensory neuropathy

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Abstract

Polymorphisms in *P2X4R* and *CAMKK2* associate with susceptibility to HIV-associated sensory neuropathy (HIV-SN) – a condition likely mediated by TNFα. As single nucleotide polymorphisms (SNPs) and haplotypes of *CAMKK2*, and a neighbouring gene *P2X4R,* mark susceptibility to HIV-SN in South Africans living with HIV, we examined the relationship between *P2X4R* and *CAMKK2* genotypes and TNFα production. Peripheral blood mononuclear cells from 129 healthy donors were stimulated with killed *Escherichia coli,* and concentrations of soluble TNFα were assessed. Their DNA was genotyped for 22 SNPs in *P2X4R* and *CAMKK2*. Three SNPs within *P2X4R* and two SNPs within *CAMKK2* influenced concentrations of TNFα, but these SNP did not associate with risk for HIV-SN. This incongruence may reflect differences in *P2X4R* haplotypes present in Africans and Europeans. However some *CAMKK2* haplotypes were found in both populations, so *CAMKK2* polymorphisms may impact upon HIV-SN via effects of the protein on pathways other than TNFα.

150 words

1. Introduction

HIV-associated sensory neuropathy (HIV-SN) is a neurological complication occurring in up to 60% of HIV+ individuals. It is a length-dependant disease predominately affecting the nerve fibres that innervate the distal limbs, particularly the feet [1]. Symptoms include pain, burning, and numbness, which impact an individual's quality of life and work capabilities [2,3]. Susceptibility has been linked to single nucleotide polymorphisms (SNPs) and haplotypes in the *P2X4R* and *CAMKK2* genes in patients of African descent [4]. However genetic analyses, post-mortem studies in humans, and animal models implicate tumour necrosis factor alpha (TNFα) in the underlying pathology [5,6,7,8]. We seek a link between TNFα, *P2X4R* and *CAMKK2*.

CAMKK2 and *P2X4R* are contiguous genes located in a region of high linkage disequilibrium on chromosome 12. *CAMKK2* encodes calcium/calmodulin-dependant protein kinase kinase 2 (CaMKK2), a protein with roles in cellular metabolism, neuronal repair and inflammation [9,10]. Little is known about the expression of CaMKK2 in immune cells, but it appears, at least in mice, to be limited to monocytes and macrophages. CaMKK2 activation by intracellular Ca^{2+} propagates CaM kinase signalling cascades, including CaM kinase IV (CaMKIV)-induced TNFα production, via p38-MAPK and Activation Factor 1. Indeed, ablation of *CAMKK2* in macrophages from knockout mice diminished toll-like receptor 4 (TLR4) signalling after stimulation with lipopolysaccharide (LPS), impairing synthesis of inflammatory chemokines and cytokines including TNFα [9].

P2X4R encodes a purinergic receptor 4 (P2X4R) which is a ligand-gated ion channel implicated in inflammatory signalling and synaptic transmission in the central nervous system. P2X4R is abundant in macrophages and microglia, where tissue insults trigger release of extracellular adenosine triphosphate (ATP) and activate P2X4R at the cell surface, initiating ion permeability. Activation of P2X4R triggers TNFα production via the p38-MAPK cascade or Brain Derived Neurotrophic Factor (BDNF) [11]. P2X4R expression was upregulated in microglial cultures after stimulation with LPS, placing the gene in a TLR4-TNFα pathway [12]. It is plausible that SNP affecting a TLR4-TNFα pathway may affect HIV-SN as TLR4-null mice exhibited attenuation of neuropathic-like hypernociception [13]. This is tested here using cells stimulated with *E.coli.*

We address the possibility that the observed associations between polymorphisms in *CAMKK2* and *P2X4R* and HIV-SN [4] may be mediated through TNFα. Overall CAMKK2 exhibited the strongest associations with HIV-SN, with two SNPs and six haplotypes predicting SN status in South Africans of African descent. Using immunohistochemistry, we confirmed that cultured CD14+ macrophages

isolated from human PBMC express P2X4R and CaMKK2 (Supplement 1). Here we explore whether genotypes associated with HIV-SN align with variations in TNFα production *in vitro.*

2. Materials and Methods

Healthy adult donors (n=129) declaring European descent were recruited in Western Australia with approval from the Royal Perth Hospital Human Research Ethics Committee. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Hypaque gradients and cryopreserved. $5x10^5$ PBMC were stimulated with $1x10^7$ cfu/ml heat-killed *E. coli* and incubated at 37°C in 5% CO2 for 4, 8 or 24 hours, in parallel with unstimulated PBMC. Soluble TNFα concentrations in the supernatants were measured using Duoset ELISA Development System (R&D Systems, Minneapolis, MN) [14]. DNA was extracted using QIAmp DNA mini Blood Kits (QIAGEN, Valencia, CA) and typed using OpenArray SNP kits (Thermo Fisher Scientific, Waltham, MA), designed to assess SNPs in *P2X4R* and *CAMKK2* [4]. The major and minor alleles in the European population are denoted 1 and 2 (respectively). SNPs outside Hardy-Weinberg Equilibrium or with no call outputs were excluded from analysis. Haplotypes with a frequency greater than 1% were derived from the genotypes using fastPHASE [15].

3. Results

Median (range) TNFα concentrations in unstimulated cultures were 0.02 (0.00-0.72), 0.02 (0.00-1.6) and 0.09 (0.01-2.1) ng/ml at 4, 8 and 24 hours. As these values are low, the effect of genotype was determined in stimulated cultures, where TNFα concentrations were 1.3 (0.01-5.4), 1.4 (0.00-10.8) and 1.4 (0.01-4.5) ng/ml after 4, 8 and 24 hours. As these values do not rise over time, data collected at 4 hours was selected as the best measure of *de novo* synthesis (Table 1).

Carriage of the P2X4R minor alleles of rs2686387 and rs10849860 associated with an increased concentration of TNFα (p=0.006 and 0.034) while rs7298368 was associated with a decrease (p=0.002), supporting an effect of the gene on a TLR4-TNFα pathway. Accordingly rs2686387, which showed a weak association with HIV-SN in South Africans, is classified as an expression quantitative trait locus (eQTL) of *P2X4R* in whole blood and tibial arteries, but rs7298368 and rs10849860 are not [16]. However, the "high TNFα" allele 2 of rs2686387 aligned with resistance to HIV-SN. We therefore considered the possibility that rs2686387 may have different haplotypic associations in populations of African and European descent. These analyses were done after adjustment of the South African data because the major and minor alleles rs2686387, rs11065504, rs1718120 and rs3817190 were reversed in the African population relative to Australian Caucasians. FastPHASE derived five haplotypes in the Australian Caucasian population, none of which occurred in the South African population (Table 2). As such, the haplotypes containing rs2686387, rs7298368 and rs10849860 must

be different. This leaves open the possibility that the SNPs affecting TNFα responses in Caucasians may affect neuropathy in Caucasians.

In Caucasians, one P2X4R haplotype associated with increased TNFα levels and contained rs2686387 and rs10849860. A second haplotype contained these SNP but occurred in only 2% of the population thus no association could be determined. Another haplotype which occurred in 15% of this population contained rs2686387 but not rs10849860 and did not associated with TNFα. This suggests that carriage of the minor alleles of both rs2686387 and rs10849860 are necessary to generate an effect on TNFα production.

For *CAMKK2,* carriage of minor alleles of rs11065504 and rs7314454 had small and opposing effects on TNFα concentrations, but neither SNP associated with HIV-SN in South Africans. Rs11065504 is located between *P2X4R* and *CAMKK2* so it may impact expression of either gene. Carriage of the minor allele of this SNP is reported as an eQTL in whole blood, tibial arteries and tibial nerves for P2X4R but not CaMKK2 expression [16]. The three *CAMKK2* SNPs associated with HIV-SN did not affect concentrations of TNFα detected in stimulated cultures at 4 hours (Table 1), so we again considered the possibility that *CAMKK2* haplotypes may be different in populations of African and European descent. FastPHASE analyses of the Caucasian genotypes generated 18 haplotypes with frequencies >1%. While eight of these are also observed in the South African population, the haplotypes which contain more than one minor allele associated with HIV-SN do not occur in both populations (Table 2). Overall we cannot determine whether haplotypic differences explain our failure to align the effects of individual SNPs on TNFα concentrations and HIV-SN.

A CAMKK2 haplotype found Caucasians and South Africans at 2 and 4% (respectively) was significantly associated with reduced TNFα levels (p=0.04). Accordingly this haplotype contained the minor allele of rs11065504 and the major allele of rs7314454, which were associated with low TNFα levels. However this combination of SNPs is found in five other haplotypes that had no interesting associations, so the causative SNP may lie outside the panel investigated in this study.

4. Discussion

It is plausible that the SNP tested affect aspects of TNFα production that are not modelled by *in vitro* stimulation of PBMC. Alternatively, their impact on HIV-SN may reflect another role of CaMKK2. For instance, another SNP significantly associated with HIV-SN, rs2686367, is located in the 5'UTR of *CAMKK2* (Goullee et al., 2016), an area containing consensus DNA for transcription factors which

regulate neuropoesis (Racioppi and Means, 2009). Furthermore, rs2686367 is classified as an eQTL of *CAMKK2* expression in tibial nerves [16], so this SNP may impact protein function in neuron repair.

In view of the data presented here, we cannot exclude the possibility that the strong link between *CAMKK2* polymorphisms and HIV-SN may result from interactions between the expressed protein and neurons. The high genetic variability of *P2X4R* and the effect of several SNP on TNFα concentrations leaves open the possibility that P2X4R may impact HIV-SN via a TLR4-TNFα pathway. It will be a challenge moving forward to exploit variations in haplotypes carried in different populations to identify SNPs critical to disease pathogenesis.

5. Acknowledgements

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6. References

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Table 1. Three SNPs in *P2X4R* and 2 in *CAMKK2* affect TNFα levels in cultures stimulated with killed *E. coli*

SNP ID[a]	HIV-SN[b]	1,1	1,2	2,2	P-Value[c]		
					1,1 vs 1,2	1,1 vs 2,2	1,2 vs 2,2
			P2X4R				
rs2686387[d] C/G (0.30)[e]	P>0.20 RR=0.70	0.95[f] (0.01-5.37) n = 61	1.85 (0.03-4.90) n = 54	1.30 (0.41-4.00) n = 10	**0.006**	0.30	0.30
rs2303998 G/A (0.03)	-[g]	1.27 (0.59-4.00) n = 118	1.55 (0.01-5.37) n = 8		0.38[h]		
rs7298368 C/T (0.18)	P>0.20 RR=0.51	1.25 (0.01-5.37) n = 86	1.68 (0.03-4.90) n = 33	0.74 (0.03-1.19) n = 6	0.07	0.16	**0.024**
rs25643 T/C (0.40)	P>0.20 RR=0.89	0.98 (0.01-5.37) n = 43	1.52 (0.03-4.90) n = 64	1.50 (0.03-4.00) n =1 9	0.29	0.25	0.80
rs10849860 T/C (0.13)	P>0.20 RR=0.92	0.97 (0.01-5.37) n = 94	1.70 (0.05-3.99) n = 33		**0.034**		
rs11608486 T/C (0.14)	P>0.20 RR=0.89	0.90 (0.00-5.37) n = 86	1.05 (0.00-4.00) n = 32		0.92		
rs7961979 C/A (0.11)	P>0.20 RR=1.11	1.04 (0.00-5.37) n = 93	0.99 (0.00-3.26) n = 26		0.74		
			CAMKK2				
rs1653587 A/G (0.07)	P=0.12 RR=1.80	1.29 (0.01-5.37) n = 107	1.30 (0.03-4.00) n = 18		0.52		
rs1653588 T/A (0.06)	P>0.20 RR=1.61	1.27 (0.01-5.37) n = 112	1.68 (0.03-3.17) n = 15		0.58		
rs11065502 G/C (0.16)	P>0.20 RR=1.01	0.95 (0.00-5.37) n = 86	1.10 (0.00-4.00) n = 38		0.97		
rs11065504[d] C/G (0.35)	P>0.20 RR=0.00	1.38 (0.03-5.37) n = 53	1.49 (0.01-4.90) n = 52	0.58 (0.01-4.72) n = 17	0.65	**0.04**	0.15
rs7975295 T/C (0.12)	P=0.007 RR=0.68	1.31 (0.01-5.37) n = 93	1.43 (0.03-4.90) n = 25		0.75		
rs2686344 C/T (0.28)	P=0.018 RR=1.67	1.21 (0.03-4.72) n = 67	1.273 (0.01-3.86) n = 50	1.30 (0.05-5.37) n = 11	0.36	0.98	0.65

rs1560568 G/A (0.11)	P=0.023 RR=0.71	1.29 (0.01-5.37) n = 103	1.43 (0.03-4.90) n = 25		0.64		
rs7314454 C/T (0.12)	P>0.20 RR=0.56	0.98 (0.01-5.37) n = 90	1.76 (0.03-4.00) n = 27		**0.017**		
rs1718120[d] G/T (0.50)	P>0.20 RR=0.00	1.05 (0.00-4.00) n = 26	1.02 (0.00-4.72) n = 60	0.60 (0.00-5.37) n = 25	0.57	0.40	0.88
rs3817190[d] A/T (0.37)	P=0.19 RR=1.91	1.29 (0.01-5.37) n = 39	1.45 (0.03-4.72) n = 44	1.36 (0.15-4.00) n = 14	0.76	0.49	0.33

a. In chromosomal order

b. Association with carriage of the minor allele and HIV-SN in South Africans [4]
P values (chi^2 tests) and Relative Risk (RR) are shown.

c. Mann Whitney statistics comparing concentrations of TNFα between genotypes

d. The major and minor alleles of these SNP were reversed in the African population relative to Australian Caucasians. The minor allele of rs2686387 in P2X4R associated weakly with increased risk of HIV-SN in univariate analyses (p=0.15) [4]. Rs11065504, rs1718120 and rs3817190 did not affect HIV-SN in African patients.

e. Major/minor allele (minor allele frequency) from samples successfully genotyped

f. Median (range) concentrations of TNFα (ng/ml) in culture at 4 hours

g. Allele 2 was not found in patients with HIV-SN

h. (1,2) and (2,2) were merged when <5 individuals carried the (2,2) genotype

Table 2. One *P2X4R* and *CAMKK2* haplotype affected TNFα levels in cultures stimulated with killed *E. coli*

Haplotype Sequence[a]	South African[b]	Australian Caucasian[c]	TNFα levels[d] Haplotype Absent	Haplotype Present	P-value[e]
			P2X4R		
1111111	0%	55%	1.10 (0.03-4.00) n = 101	1.39 (0.01-5.37) n = 28	0.47
2122111	0%	15%	1.26 (0.01-5.37) n = 93	1.51 (0.03-4.90) n = 36	0.24
1112122	0%	13%	1.29 (0.01-5.37) n = 104	1.23 (0.03-3.26) n = 25	0.68
2112211	0%	12%	0.98 (0.01-5.37) n = 106	2.08 (0.06-3.00) n = 23	**0.01**[f]
2212221	0%	2%	1.28 (0.01-5.37) n = 123	1.55 (0.59-4.00) n = 6	0.56
			CAMKK2		
1111121111	0%	16%	1.14 (0.01-4.90) n = 100	1.50 (0.03-5.37) n = 29	0.33
1112111111	0%	12%	1.29 (0.01-5.37) n = 92	0.95 (0.01-4.90) n = 37	0.56
1112111122	18%	12%	1.29 (0.01-5.37) n = 99	1.39 (0.03-4.72) n = 30	0.52
1121111122	0%	11%	1.29 (0.01-5.37) n = 108	1.23 (0.03-3.18) n = 21	0.77
1111212111	0%	7%	1.28 (0.01-5.37) n = 113	1.62 (0.09-4.90) n = 16	0.56
1111111111	0%	7%	1.23 (0.01-5.37) n = 119	2.08 (0.05-2.84) n = 10	0.32
2211111121	3%	4%	1.29 (0.01-5.37) n = 121	1.23 (0.03-2.71) n = 8	0.84
1111111222	1%	3%	1.26 (0.01-5.37) n = 125	1.89 (1.68-2.49) n = 4	0.24
1111121121	1%	2%	1.28 (0.01-5.37) n = 125	1.68 (0.60-3.00) n = 4	0.47
1111111122	0%	2%	1.28 (0.01-5.37) n = 125	2.37 (0.15-3.26) n = 4	0.32
1112121111	0%	2%	1.30 (0.01-5.37) n = 127	0.35 (0.28-0.42) n = 2	0.18
1111121222	2%	2%	1.26 (0.01-5.37) n = 126	1.67 (0.05-3.86) n = 6	0.40
1112121122	4%	2%	1.33 (0.01-5.37) n = 123	0.03 (0.01-0.55) n = 6	**0.0003**[g]
1112111121	7%	2%	1.33 (0.01-5.37) n = 125	0.49 (0.07-1.18) n = 4	0.13
2211111111	0%	1%	1.29 (0.01-5.37) n = 124	1.50 (0.59-3.03) n = 5	0.46
1111212121	3%	1%	1.29 (0.01-5.37) n = 126	0.90 (0.03-1.68) n = 3	0.36
1121111121	0%	1%	1.30 (0.01-5.37) n = 127	0.94 (0.62-1.26) n = 2	0.64

1121111222	0%	1%	1.27 (0.01-5.37) n = 124	2.76 (0.74-4.00) n = 5	0.08

[a.] Defined by alleles of SNPs in the order shown in Table 1

[b.] The fastPHASE haplotype frequency in the South African HIV^{+} population [4]

[c.] The fastPHASE haplotype frequency in this Australian Caucasian population

[d.] Median (range) concentrations of TNFα (ng/ml) in culture stimulated with killed *E. coli* at 4 hours

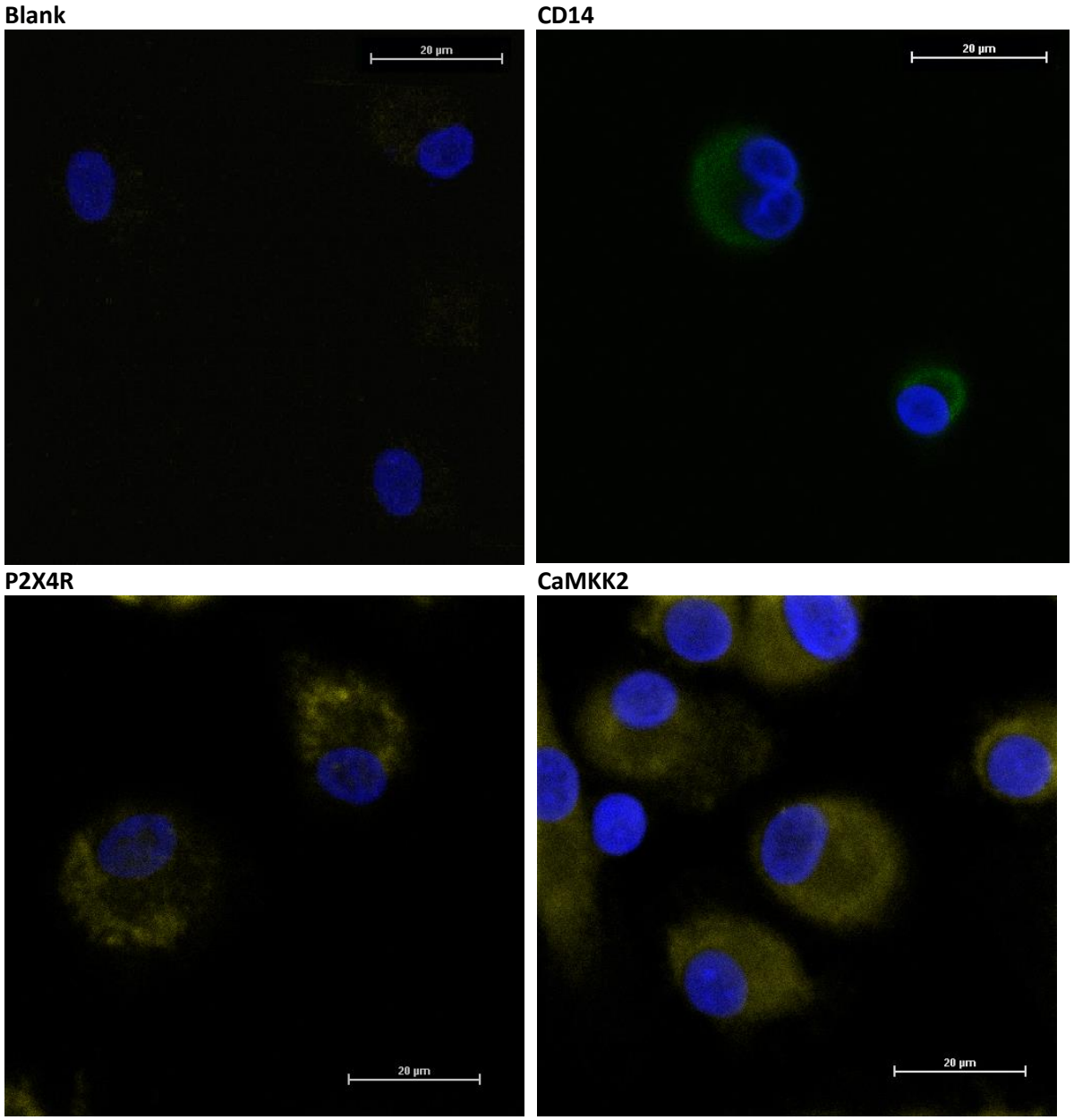
[e.] Mann Whitney statistics comparing concentrations of TNFα between genotypes

[f.] Includes minor alleles of rs2686387 and rs10849860 associated with increased TNFα

[g.] Includes the minor allele of rs11065504 and major allele of rs7314454 associated with reduced TNFα

Figures

SUPPLEMENT 1.



Supplementary Figure 1. CD14+ macrophages express CaMKK2 and P2X4R

Macrophages cultures isolated from human PBMC were stained with FITC labelled anti-CD14 (mouse 1:20; BioLegend, CA, USA), or unlabelled anti- CaMKK2 (goat 1:200; AbCam, Cambridge, UK) or P2X4R (goat 1:200; AbCam). The biotinylated secondary antibody donkey anti-goat IgG (AbCam, Cambridge, UK) was used at 1:100 and detected using Alexa Fluor 647 labelled Streptavidin (1:100, Life Technologies, CA, USA). Images were viewed and enhanced with Nikon A1 confocal microscope and NIS-Elements Viewer (Nikon Instruments, NY, USA).