Genetic inactivation of calpain-1 attenuates pain sensitivity in a humanized mouse model of sickle cell disease

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SUPPLEMENTARY DATA

METHODS

Generation of calpain-1 knockout Townes sickle cell mice: Townes sickle (SS) mice were obtained from the Jackson Laboratory, and backcrossed with our calpain-1 knockout C57BL/6 (CKO) mice to generate humanized calpain-1 knockout sickle (SSCKO) mice. Humanized (AA) or wild type C57BL/6 mice were used as controls. Male and female mice were used except otherwise indicated. The Tufts University Institutional Animal Care and Use Committee approved all animal procedures.

Hematological analysis and Casein Zymography: (Table 1) Complete blood counts were measured by ADVIA 2120 (Siemens). Casein zymography of calpain-1 activity in peripheral blood RBCs was performed as described (Raser KJ, Posner A, Wang KK. Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch Biochem Biophys*. 1995;319(1):211-216).

Hypoxia/reoxygenation treatment: Mice were exposed to 3 hours of hypoxia (10.5% O_2, 89.5% N_2), followed by 4 hours of reoxygenation at room air (22% O_2) prior to indicated experiments. For hypoxia/reoxygenation- evoked pain, mice were exposed to 3 hours of hypoxia (8% O_2, 92% N_2) followed by 1 hour at room air.

Behavioral pain testing: Chronic and H/R-evoked acute pain (deep/musculoskeletal pain, mechanical hyperalgesia, and thermal sensitivity to heat and cold) were measured as previously described (Vincent L, Vang D, Nguyen J, et al. Mast cell activation contributes to sickle cell pathobiology and pain in mice. *Blood*. 2013;122(11):1853-1862; Kohli DR, Li Y, Khasabov SG, et al. Pain-related behaviors and neurochemical alterations in mice expressing sickle hemoglobin: modulation by cannabinoids. *Blood*. 2010;116(3):456-465).

Statistical analyses: In behavioral pain measurements, differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test (GraphPad Prism v 5.0a). All other analyses were conducted using unpaired t tests (Microsoft Excel 2010). Data are presented as mean ± SEM except otherwise stated. A P value of < 0.05 was considered significant.

Table 1. Hematological parameters

Parameter	AA	SS	SSCKO
WBC (K/µL)	6.7 ± 1.6	26.3 ± 2.3**	37.5 ± 5.4
RBC (M/µL)	11.0 ± 0.3	6.1 ± 0.3$^{\#\#}$	6.1 ± 0.3
HGB (g/dL)	10.8 ± 0.4	5.5 ± 0.5**	6.2 ± 0.3
CHB (g/dL)	10.8 ± 0.4	6.9 ± 0.3**	7.3 ± 0.3
HCT (%)	43.3 ± 1.4	30.2 ± 1.3**	32.3 ± 1.3
MCV (fL)	39.2 ± 0.3	49.8 ± 0.6$^{\#\#\#}$	53.3 ± 1.0§
MCH (pg)	9.7 ± 0.1	9.0 ± 0.7	10.1 ± 0.1
MCHC (g/dL)	24.8 ± 0.2	18.2 ± 1.4*	19.0 ± 0.2
CHCM (g/dL)	24.9 ± 0.2	22.9 ± 0.2**	22.7 ± 0.2
CH (pg)	9.6 ± 0.1	11.1 ± 0.1$^{\#}$	11.9 ± 0.1§
RDW (%)	22.6 ± 1.2	28.7 ± 0.4**	26.0 ± 0.6§
HDW (g/dL)	2.7 ± 0.06	3.7 ± 0.08$^{\#}$	3.6 ± 0.1
RETIC (%)	6.5 ± 0.5	44.0 ± 1.6$^{\Psi}$	50.8 ± 0.8§

Data are mean ± SEM. Complete blood counts were measured in peripheral blood by ADVIA 2120. Unpaired T-test; $n = 5 – 9$ mice per group. *$P < 0.01$ vs. AA; **$P < 0.0001$ vs. AA; $^{\#}P < 0.00001$ vs. AA; $^{\#\#}P < 0.000001$ vs. AA; $^{\#\#\#}P < 0.0000001$ vs. AA; $^{\Psi}P < 0.000000001$ vs. AA; $^{¥}P < 0.05$ vs. AA; $^{\S}P < 0.01$ vs. SS; $^{\$}P < 0.01$ vs. SS.