Targeting Peptidyl Arginine Deiminase 4 and NADPH Oxidase Pathways Regulates Neutrophil Dependent Thrombo-inflammation

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Introduction/Background & aims: Excessive neutrophil extracellular trap (NET) production is associated with the pathogenesis of thrombo-inflammatory diseases e.g. sickle cell disease (SCD), and is catalyzed by the enzyme Peptidyl Arginine Deiminase-4 (PAD4). Neutrophil reactive oxygen species (ROS), especially NADPH-oxidase (NOX) interacts with PAD4 and is critical for its function. Here, we studied the critical role of neutrophilic NOX in NET formation associated with thrombo-inflammation.

Method/Summary of work: In-vitro. Neutrophils were isolated from age-matched control volunteers and SCD patients (institutional review board of LSUHSC-S approved the study, conducted in accordance to the Declaration of Helsinki). NET assays were performed (Sytox green and citrullinated histone-3 (H3cit+) neutrophil assays) using two distinct stimuli; ionomycin [4 μM] (for maximal NET production) and the Protein-kinase C activator, phorbol-12-

myristate-13-acetate (PMA) [100 nM] (which elicits NET production in a ROS-dependent manner). GSK484 [10 μM] (PAD4 inhibitor), and VAS3947 [5 μM] (NOX inhibitor) were used as pharmacological tools to target PAD4 and NOX respectively. In-vivo. Male (10–12 weeks) C57BL/6 (control) and sickle-transgenic mice (STM) were anaesthetized with ketamine (150 mg/kg) and xylazine (7.5 mg/kg) i.p. A photoactivation thrombosis model (light/dye) was performed and mice were treated with PAD4 [10 μM] and NOX [5 μM] to study their effect on cerebral thrombo-inflammatory responses. Animal experiments complied with ARRIVE guidelines and followed LSUHSC-S IACUC. Statistical significance was determined using ANOVA followed by Bonferroni posthoc test, or Student's t test. Differences were considered statistically significant at $p < 0.05$. Data are mean±SEM of $n = 6–8$ mice/group and $n \geq 5$ independent experiments.

Results/Discussion: Results. In-vitro. Targeting PAD4 and NOX, alone or in combination, in neutrophils obtained from control volunteers significantly inhibited ionomycin dependent H3cit+ neutrophils vs. ionomycin stimulated neutrophils ($p < 0.05$. Table-1). However, PMA-dependent H3cit+ neutrophils were only inhibited by targeting NOX vs. PMA stimulated neutrophils ($p < 0.05$), and not PAD4 alone (Table-1). These results suggest PMA stimulation either bypasses PAD4 activation, or it employs an accessory ROS-dependent PAD4-independent pathway for H3Cit + neutrophil production. Invivo. In control mice, targeting PAD4 and NOX was able to prolong cerebral arteriolar blood-flow cessation times (minutes):

21.89± 1.42 (vehicle) vs. 51.57± 2.26* (GSK484) or 47.25 ± 4.78* (VAS3947) (*p < 0.05 vs. vehicle). Blood flow cessation times were much faster in STM vs. control mice: 14.93 ± 0.77 vs. 21.89± 1.42*minutes (*p < 0.05 vs. STM) respectively. Moreover, targeting PAD4 and NOX in a clinical model of thrombo-inflammation (i.e. in STM) also increased cerebral blood-flow cessation times (minutes): 14.93 ± 0.77 (vehicle) vs. 28.16± 1.76* (GSK484) or 19.89± 1.51* (VAS3947) (*p < 0.05 vs. vehicle STM), although the effect was not as pronounced as that observed in control mice.

Conclusion(s): These data demonstrate PAD4 and NOX play a significant role in neutrophil driven thrombo-inflammation. Pathological NETosis results in excessive H3cit+ neutrophils which can be attenuated by targeting PAD4 and NOX production, and may help to explain the augmented cerebral thrombosis responses in-vivo. Overall, this study demonstrates targeting PAD4 and NOX may provide new and effective therapeutic possibilities for thrombo-inflammatory conditions such as SCD.

TABLE 1 Inhibition of human NETs

	Control	Ionomycin	PMA	Ionomycin +GSK484	Ionomycin +VAS3947	PMA +GSK484	PMA +VAS3947
Control Volunteers	18.02 ±1.78%	66.33 ±8.59%	51.68 ±7.52%	19.44* ±4.34%	27.92* ±7.56%	51.26 ±8.37%	11.38$ ±4.12%
SCD	31.25 ±6.04%	57.13 ±3.80%	50.12 ±11.13%	34.20 ±6.70%	19.14# ±4.01%	46.30 ±8.66%	14.11Φ ±4.39%

*$p < 0.05$ vs. ionomycin treated control neutrophils $p < 0.05 vs. PMA treated control neutrophils

#$p < 0.05$ vs. ionomycin treated SCD neutrophils

Φ$p < 0.05$ vs. PMA treated SCD neutrophils

Key: NETs were quantified as the percentage of $H3cit^+$ stained DNA over total number of neutrophils (DAPI stained) in a double blinded fashion.