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Targeting AnxA1/Fpr2/ALX Regulates Neutrophil Function Promoting Thrombo-Inflammation Resolution in Sickle Cell Disease

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Abstract:
Neutrophils plays a crucial role in the intertwined processes of thrombosis and inflammation. Altered neutrophil phenotype may contribute to inadequate resolution which is known to be a major pathophysiological contributor of thrombo-inflammatory conditions such as Sickle Cell Disease (SCD). The endogenous protein Annexin A1 (AnxA1) facilitates inflammation resolution via Formyl Peptide Receptors (FPRs). We sought to comprehensively elucidate the functional significance of targeting neutrophil dependent AnxA1/FPR2/ALX pathway in SCD. Administration of AnxA1 mimetic peptide $AnxA1_{Ac2-26}$ ameliorated cerebral thrombotic responses in Sickle transgenic mice via regulation of FPR2/ALX (a fundamental receptor involved in resolution) pathway. We demonstrated direct evidence that neutrophils with SCD phenotype play a key role in contributing to thrombo-inflammation. In addition, $AnxA1_{Ac2-26}$ regulated activated SCD neutrophils through protein kinase B (Akt) and extracellular signal-regulated kinases (ERK1/2) to enable resolution. Herein, we present compelling conceptual evidence that targeting the AnxA1/FPR2/ALX pathway may provide new therapeutic possibilities against thrombo-inflammatory conditions such as SCD.

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Targeting AnxA1/Fpr2/ALX Pathway Regulates Neutrophil Function Promoting Thrombo-Inflammation Resolution in Sickle Cell Disease

Short title: AnxA1 promotes thrombo-inflammation resolution in Sickle Cell Disease

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Key Points:

1. $AnxA1_{Ac2-26}$ plays a key role in mitigating neutrophil-dependent thrombo-inflammatory responses in Sickle Cell Disease.
2. Targeting the AnxA1/Fpr2/ALX pathway attenuates and protects against thrombo-inflammatory events by switching the pathological neutrophil phenotype from a pro-NETotic to pro-apoptotic, thereby driving resolution.

Abstract

Neutrophils plays a crucial role in the intertwined processes of thrombosis and inflammation. Altered neutrophil phenotype may contribute to inadequate resolution which is known to be a major pathophysiological contributor of thrombo-inflammatory conditions such as Sickle Cell Disease (SCD). The endogenous protein Annexin A1 (AnxA1) facilitates inflammation resolution via Formyl Peptide Receptors (FPRs). We sought to comprehensively elucidate the functional significance of targeting neutrophil dependent AnxA1/FPR2/ALX pathway in SCD. Administration of AnxA1 mimetic peptide $AnxA1_{Ac2-26}$ ameliorated cerebral thrombotic responses in Sickle transgenic mice via regulation of FPR2/ALX (a fundamental receptor involved in resolution) pathway. We demonstrated direct evidence that neutrophils with SCD phenotype play a key role in contributing to thrombo-inflammation. In addition, $AnxA1_{Ac2-26}$ regulated activated SCD neutrophils through protein kinase B (Akt) and extracellular signal-regulated kinases (ERK1/2) to enable resolution. Herein, we present compelling conceptual evidence that targeting the AnxA1/FPR2/ALX pathway may provide new therapeutic possibilities against thrombo-inflammatory conditions such as SCD.

Introduction

Neutrophils, the most abundant immune cells (contributing to 60-70% of the leukocyte population) and one of the most important effector cells of the immune system, play crucial roles in inflammation resolution. Inflammation is interconnected with thrombosis, with one begetting the other, leading to a pro-thrombo-inflammatory state[1,2] that is associated with a number of diseases including sickle cell disease (SCD),[3] diabetes,[4] and cancer.[5] These chronic thrombo-inflammatory diseases often result in severe acute cardiovascular complications (acute ischemic stroke [AIS], pulmonary embolism and microvascular angiopathies[6]) which are the most frequent cause of morbidity and mortality worldwide. [6,7]

Sickle Cell Disease (SCD) is an inherited autosomal recessive disorder resulting from a single amino acid substitution in the hemoglobin β chain.[8] The pathophysiology of SCD is characterized by a relentless pro-thrombo-inflammatory state, which enables a heightened propensity for ischemic events such as AIS. Despite these patients being more susceptible to ischemic events and having poorer outcome post-AIS, the mechanisms remain poorly understood. However, these detrimental effects may be caused by inadequate class switching of endogenous pro-resolving mediators.[9,10] Thus, SCD provides a unique model to study a defective resolution process to ascertain whether engaging resolution pharmacology will be an optimal therapeutic approach to target thrombo-inflammation.[11,12]

Under homeostatic conditions, inflammation resolution is orchestrated by a tightly controlled series of endogenous biosynthetic mediators (e.g. Annexin A1 [AnxA1] and its biologically active N-terminal domain, peptide $AnxA1_{Ac2-26}$,[13] aspirin triggered lipoxin A_4 [15(R)-epi-LXA_4

(ATL)])[14] and protective pro-resolution pathways (e.g. formyl peptide receptor-[FPR]-pathway),[15] However, these endogenous biosynthetic circuits may be hampered in chronic thrombo-inflammatory diseases. Both AnxA1 and $AnxA1_{Ac2-26}$ are known to exert their anti-inflammatory and pro-resolving actions in acute and chronic inflammation by engaging with FPR2/ALX.[13,15,16] Thus, exploring AnxA1/FPR2/ALX pathways in disease states characterized by chronic uncontrolled thrombo-inflammation, may provide a viable therapeutic strategy to promote resolution, affording protection.

Using genetic and pharmacological approaches, along with clinical samples, we present novel and compelling data that $AnxA1_{Ac2-26}$ plays a key role in mitigating neutrophil-dependent thrombo-inflammatory responses in the cerebral microvasculature via AnxA1/FPR2/ALX pathway. Furthermore, we demonstrate that exogenous administration of $AnxA1_{Ac2-26}$ regulates pro-thrombotic neutrophil responses without affecting physiological responses, through protein kinase B (Akt) and extracellular signal-regulated kinases (ERK1/2),[17,18] which act as molecular switches to transform the neutrophil phenotype from a pro-NETotic to pro-apoptotic phenotype thereby driving resolution. These compelling data demonstrate the propensity for therapeutic strategies based on Resolution Biologics for the management of thrombo-inflammatory complications.

Material and Methods

See Online Supplement for in-depth methods.

Drugs, reagents and antibodies

For *in-vivo* experiments, vehicle (saline), Annexin A1 (AnxA1) mimetic peptide Ac2-26 ($AnxA1_{Ac2-26}$, Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Cambridge Research Biochemicals) 100 µg/mouse,[19] Boc2 (N-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe, MP Biomedicals, Cambridge, UK) 10 µg/mouse,[19] and WRW4 (55 µg/mouse) (EMD Biosciences Inc) were administered (100 µl) intravenously (i.v.) at the start of cerebral reperfusion.[20]

For *in-vitro* experiments, vehicle 1X Phosphate buffered saline (PBS) (Life Technologies) $AnxA1_{Ac2-26}$ (30 µM), Boc2 (10 µM),WRW4 (10 µM),[21] Akti-1/2 (10 µM),[18] U0126 (10 µM) (Tocris), Caspase-3 inhibitor Z-DEVD-FMK (20 µM) (R&D systems, Minneapolis, MN, USA) were used as pharmacological tools NETs were induced by ionomycin (4 µM) (Life Technologies). NET specific stains include neutrophil elastase rabbit anti-NE (1:200) (Abcam), histone H3 mouse anti-H3Cit (1:200) (Cell Signaling Technology), and (Sigma-Aldrich).

Animals

Male control and Sickle Cell Transgenic mice (STM. Townes) (Homozygous at the *Hba* locus for the hα mutation [$Hba^{tm1(HBA)Tow}$] and homozygous at the *Hbb* locus for the -383 γ-β^A mutation [$Hbb^{tm3(HBG1,HBB)Tow}$]) were purchased from Jackson Laboratory (Bar Harbor). Animal Care and Use Committee of LSUHSC-S approved experimental procedures performed on the mice. All studies were performed blinded and randomized and all studies complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines.

Human samples

The study was approved by the institutional review board of the LSUHSC-S (STUDY00000572 and STUDY00000261) and conducted in accordance with the Declaration of Helsinki. After signed consent was obtained, blood was taken from control volunteers (18-52 years old, twenty-six males, eighteen females) and SCD patients (18-52 years old, nineteen males, twenty-eight females). All but one of the SCD patients were of HbSS (homozygous hemoglobin S) genotype. One patient was HbSC (sickle hemoglobin C disease) genotype. SCD patients were recruited upon routine clinical visits at the Feist-Weiller Cancer Center at LSUHSC-S. All SCD patients were on chronic hydroxyurea therapy and blood was obtained just before exchange transfusion. Hydroxyurea was started at 15 mg per kilogram of body weight per day and then escalated by 5 mg per kilogram every 12 weeks until the maximum tolerated dose was achieved on the basis of peripheral blood counts. Patients were on partial exchange transfusion every two weeks. Patients with acute infection or other chronic blood borne diseases (HIV, Hepatitis B/C) were excluded from the study. Demographic and clinical characteristics of controls and SCD patients are included in ~~(Hemodynamic parameters [*Supplementary Table 3*].)~~ *Supplementary Table 3*.

Neutrophil depletion and DNase I treatment

Neutropenia was induced using mouse anti-neutrophil serum (ANS; 1A8. 150 μg/mouse, intraperitoneally 24 hours before the experiment).[22] DNase I (2000U) was administered i.v for NET degradation.

Thrombosis

Anesthetized mice (Ketamine: Xylazine, 1:1) were kept under the microscope after jugular vein cannulation and open window craniotomy. Thrombosis in cerebral vessels was induced using the light/dye thrombosis model.[23] After 20 minutes of equilibration, 10 mg/kl of 5% FITC-dextran (150 000 MW) (Sigma-Aldrich) was injected via the femoral vein and allowed to circulate for 10 minutes. Photoactivation was initiated (excitation, 495 nm; emission, 519 nm) by exposing 100 μm of vessel length to epi-illumination with a 175-W xenon lamp (Lamda LS; Sutter) and a fluorescein filter cube (HQ-FITC; Chroma). Time was recorded when the platelet aggregates first start appearing (onset time) and the time when flow stops for 30 seconds (cessation time). 30 minutes prior to onset of thrombosis, mice were treated with vehicle, $AnxA1_{Ac2-26}$ (100 μg/mouse), Boc2 (10 μg/mouse), or WRW4 (55 μg/mouse).

ELISA for NETs

To quantify the NETs in the circulating blood, 96 well immunoassay plates (#9018, Costar) were coated with a neutrophil elastase antibody (1:250, Abcam) in 15 mM of Na_2CO_3, at pH 9.6 (250 μl per well) overnight at 4°C. The following day wells were washed three times with PBS, followed by blocking with 5% BSA for two hours at room temperature. After blocking, the wells

were washed again (three times) with PBS. 50 µl of murine plasma was added to the wells and incubated for two hours at room temperature and kept on a microplate shaker at 250 rpm. The plates were then washed (three times) with wash buffer (1% BSA, 0.05% Tween 20 in PBS). This was followed by incubation with the immunoreagent (100 µl in each well). The immunoreagent was prepared by mixing 1/20 volume of anti-DNA-Peroxidase conjugated antibody (Anti-DNA-POD) with 19/20 volumes incubation buffer (Cell Death Detection ELISA^{PLUS}, Roche) for two hours at room temperature and kept on a microplate shaker at 250 rpm. The solution was removed, and each well was rinsed three times with 250 µl of incubation buffer. Next, 100 µl of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Roche) was added and the plate was incubated for 10-20 minutes on a microplate shaker at 250 rpm. The reaction was stopped by pipetting 100 µl of ABST stop solution (ROCHE) and the absorbance was measured at 405 nm plate reader (Synergy H1, BioTek) (reference wavelength approx. 490 nm). Data was analyzed as per the manufacturer's instructions (Roche).

Murine neutrophil isolation and adoptive transfer

Murine neutrophils were isolated, kept at $5x10^6$/ml and treated with AnxA1Ac2-26 (30µM), WRW4 (10µM) or AnxA1Ac2-26+WRW4 for 10 minutes prior to injection ($5x10^5$ cells per mouse) via the jugular vein of the recipient neutropenic mouse.

Isolation of human neutrophils

Neutrophils were isolated from control donors and SCD patients using dextran (spectrum chemical) sedimentation followed by Histopaque 1077.[24]

Visualization and quantification of NETs

Neutrophils ($1x10^5$/well) were seeded on poly-l-lysine coated coverslips and were stimulated for 3hours at 37oC, 5% CO_2. The cells were fixed (10% formalin), permeabilized (0.5% Triton X-100), blocked (10% goat serum) and incubated with NET specific antibodies (neutrophil elastase rabbit anti-NE [1:200], histone H3 mouse anti-H3Cit [1:200]) and species-specific secondary antibodies, and visualized.

Annexin A1 quantification in plasma

Human and murine AnxA1 ELISA kits (MyBioSource) were used to quantify the plasma levels of AnxA1. Plasma preparation and the ELISA assay were done according to manufacturer's instructions. Results were reported as ng/ml of AnxA1 concentration in plasma of control volunteers and SCD patients or in plasma of control and STM mice.

Western blotting

Samples were immunoblotted with rabbit anti-phospho-Akt1 (1:1000), goat anti-Akt1 (1:500), rabbit anti-phospho-ERK1/2 (1:1000), or rabbit anti-ERK1/2 (1:5000) antibodies overnight followed by species-specific secondary antibodies.[25]

Cleaved caspase-3 for apoptotic cells

Neutrophils ($2x10^5$/well) were seeded on poly-l-lysine coated coverslips and treated with 1X PBS or AnxA1Ac2-26 (30µM) (3hours, 37°C, 5% CO_2). Cells were incubated with caspase-3 antibody overnight (1:500) followed by species-specific secondary antibody.

Myeloperoxidase release assay (MPO)

Neutrophils ($1x10^5$/well) were left unstimulated, ionomycin-stimulated (4μM), or pre-treated with $AnxA1_{Ac2-26}$ (30 μM) 15minutes prior pre-stimulation (3 hours at 37^oC, 5% CO_2). After 3hours, supernatant was collected, added to MPO solution, reaction terminated with H_2SO_4(2N) and absorbance read (450nm).

Chemotaxis assay

Human neutrophils tagged with Calcein-AM (Invitrogen) were left unstimulated, ionomycin-stimulated (4 μM), or pre-treated with $AnxA1_{Ac2-26}$ (30 μM) 15 minutes prior pre-stimulation. Neutrophils were added to ChemoTx® System. After 3 hours fluorescence intensity was measured (485/530nm excitation/emission) to determine neutrophil chemotaxis towards LTB_4 (10^{-6} M) or PBS (control).

Quantification of Interleukin 1 beta (IL-1β) ELISA

Human neutrophils were left unstimulated, ionomycin-stimulated (4 μM), or pre-treated with $AnxA1_{Ac2-26}$ (30 μM) 15 minutes prior pre-stimulation. After 5 hours, supernatant was collected for ELISA assay.

Data Sharing Statement

For original data, please contact felicity.gavins@brunel.ac.uk

Statistical Analysis

All data was tested to follow a normal distribution using Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lillie D'Agostino-Pearson omnibus normality test for corrected *p* value. Data that passed the normality assumption was analyzed using Student's *t*-test (two groups) or ANOVA with Bonferroni post-tests (more than two groups). Data that failed the normality assumption were analyzed using the non-parametric Mann-Whitney U test (two groups) or Kruskal-Wallis with Dunn's test (more than two groups). Analysis was performed using Graph Pad Prism 6 software (San Diego). Data are shown as mean values ± standard error of the mean (SEM). Outliers were defined as ≥ two standard deviations and have been stated in the figure legends where necessary. Differences were considered statistically significant at a value of $p < 0.05$.

Results

$AnxA1_{Ac2-26}$ ameliorates exacerbated cerebral thrombosis in SCD via Fpr2/ALX

Thrombo-inflammatory disease states such as SCD are often associated with a microcirculation that assumes a pro-inflammatory and pro-thrombotic state.[26] To validate our experimental model of thrombo-inflammation, we performed a light/dye injury model[23] (enabling visualization of thrombus formation in real-time [*Videos 1+2*]) and observed accelerated thrombus formation (decrease in blood flow cessation time) in both cerebral arterioles ($p<0.0001$) and venules ($p<0.01$) of STM vs. control mice (*Figure 1A-F*), concurring with our previous findings in a non-humanized SCD mouse model.[23]

AnxA1 and its mimetic peptides (e.g. $AnxA1_{Ac2-26}$) are recognized anti-inflammatory compounds that have shown therapeutic potential in a diverse range of disease models including e.g. ischemia reperfusion induced lung injury, acute colitis, renal transplantation, diabetic nephropathy, atherosclerosis, acute lung injury and colitis.[27] However their effects on thrombosis remain fairly unknown, although we have recently shown their attenuation of platelet responses.[16] Here, *Figure 1E+F* shows $AnxA1_{Ac2-26}$ had no effect on cerebral thrombosis when exogenously administered to control mice, but significantly increased ~~decreased~~ blood flow cessation time in cerebral arterioles ($p<0.001$) and venules ($p<0.01$) of STM mice.

Having established the anti-thrombotic effect of $AnxA1_{Ac2-26}$ in STM, we next tested whether these effects were mediated via an interaction with the FPR-pathway (especially FPR2/ALX, which is a key receptor involved in resolution and a receptor through which AnxA1 mediates its effects). *Figure 1E+F* shows the FPR pan-antagonist Boc2 blocked the protective actions of

AnxA1$_{Ac2-26}$ (p<0.001), suggesting a mechanism of action via the FPR family. To further tease out which receptor, mice were co-administered AnxA1$_{Ac2-26}$+WRW4 (specific FPR2/ALX antagonist). WRW4 blocked AnxA1$_{Ac2-26}$ afforded protection in both cerebral arterioles (p<0.001) and venules (p<0.001) thereby confirming an FPR2/ALX mechanism (*Figure 1E+F*).

Neutrophils contribute to cerebral thrombosis in STM

Compelling evidence exists demonstrating a pivotal role of neutrophils in thrombo-inflammation[28] although the role they play in context of cerebral thrombosis is less well discerned. To address this knowledge-gap we rendered STM mice neutropenic with the well-characterized anti-neutrophil serum (ANS), 1A8. Using real-time live imaging, we observed a phenotype reversal i.e. no difference in cerebral thrombosis formation between control vs. STM/ANS mice (*Figure 2B+C*), demonstrating that the cerebral microvasculature in STM mice was rendered vulnerable to thrombus formation via a neutrophil-dependent mechanism. Furthermore, ~~adoptive~~ transfer of STM, but not control, neutrophils into neutropenic STM mice (*Figure 2A*) restored the SCD-associated acceleration of thrombus formation in cerebral pial vessels (*Figure 2B+C*). These results suggest that the STM neutrophil plays a key role in mediating the accelerated cerebral thrombus formation observed in this experimental thrombo-inflammatory model.

AnxA1$_{Ac2-26}$ inhibits the thrombotic NET phenotype associated with thrombo-inflammation

Having discovered neutrophils play a significant role in cerebral thrombosis and exploiting the fact that AnxA1/FPR2/ALX pathway can mitigate these unwanted responses, we next characterized the mechanisms involved in this process. NET formation is associated with the

pathogenesis of several thrombo-inflammatory diseases e.g. in thrombi associated with deep-vein thrombosis and detected via histology in the lungs of SCD mice.[29] Here, administration of DNase (which breaks down NETs) delayed blood flow cessation in STM mice, an effect absent in control mice (*Figure 2D*).

No differences in circulating AnxA1 plasma levels in control vs. STM were quantified (*Figure 2E*), but treatment of control and STM mice with $AnxA1_{Ac2-26}$ resulted in significant reduction of neutrophil elastase (NE-DNA) complexes in STM but not control plasma ($p<0.05$) (*Figure 2F*) suggesting an anti-thrombotic mechanism and elucidating a drug-sparing effect in normal cohorts.

To test whether $AnxA1_{Ac2-26}$ anti-thrombotic effect *in-vivo* occurs by reducing the capability of neutrophils to release NETs (known contributors to thrombosis),[30] isolated neutrophils (control and STM mice) were treated with the NET-inducing stimuli ionomycin (a natural calcium ionophore) to stimulate maximal NET production with and without $AnxA1_{Ac2-26}$. Citrullinated histone-3 (H3Cit) is the most common NET biomarker that has been associated with experimental thrombosis.[31] *Figure 2G* shows $AnxA1_{Ac2-26}$ treatment significantly reduced the percentage of ionomycin-stimulated STM neutrophils that were positive for $H3Cit^+$ ($p<0.05$), an effect not observed in control neutrophils, further emphasizing the specific effect of $AnxA1_{Ac2-26}$ on neutrophils exposed to a chronic thrombo-inflammatory milieu associated with SCD.

Targeting Fpr2/ALX reduces human $H3Cit^+$ SCD neutrophils without affecting physiological responses

To translate our findings from mouse models into clinical setting, we isolated neutrophils from control volunteers and SCD patients (*Figure 3A+B*). The percentage of unstimulated H3Cit^{+}SCD neutrophils was increased compared to control neutrophils (*Figure 3C*), which was further exacerbated with ionomycin ($p<0.05$), suggesting SCD neutrophils are characterized by extensive histone citrullination potentially contributing to the pro-thrombotic phenotype. Interestingly, our SCD patients presented with reduced circulating AnxA1 plasma levels versus control volunteers, highlighting a possible defect in the resolution process as is often seen in chronic thrombo-inflammatory states (*Figure 3D*). Moreover, as observed with murine neutrophils AnxA1$_{Ac2-26}$ significantly attenuated ionomycin-induced H3Cit^{+} SCD neutrophils ($p<0.0001$) (*Figure 3E*) (no effect was observed in control volunteers). Finally, to determine the mechanistic role of FPR-family in these events, FPR pan-antagonist Boc2 was co-administered with AnxA1$_{Ac2-26}$. *Figure 3E* shows Boc2 significantly abrogated AnxA1$_{Ac2-26}$ effects, increasing H3Cit^{+} SCD neutrophil percentage ($p<0.05$). More specifically, when co-administered with AnxA1$_{Ac2-26}$, the selective Fpr2/ALX antagonist WRW4 increased the percentage of H3Cit^{+} SCD neutrophils, thus abrogating the protective actions of AnxA1$_{Ac2-26}$. These results highlight the protective effects of AnxA1$_{Ac2-26}$ on NET production were mediated through Fpr2/ALX.

Interestingly, although AnxA1$_{Ac2-26}$ modulated histone citrullination under pro-NETotic conditions, the resolving peptide did not affect other neutrophil physiological responses such as MPO release and chemotaxis (*Supplementary Figure 2A-C*). This lack of effect could be due to the fact that under pro-NETotic conditions, neutrophils are in point of no return releasing their MPO following membrane disruption. Furthermore, although IL-1β production was increased in unstimulated SCD neutrophils compared to control, it was dramatically reduced when neutrophils

were stimulated with ionomycin, with $AnxA1_{Ac2-26}$ having no effect (*Supplementary Figure 2D*). These effects may be due to the ability of ionomycin-induced NETs to produce IL-1β degrading serine proteases.[32]

$AnxA1_{Ac2-26}$ regulates the NETosis-apoptosis axis in human SCD neutrophils: Impact for therapeutic strategy against thrombosis

Due to importance of ERK and Akt kinases in NET release [17,18,33] we examined the expression of these kinases in control and SCD neutrophils (*Figures 4A*). A significant increase in ERK phosphorylation (at 15 minutes, $p<0.001$ respectively) and Akt phosphorylation (30 and 60 minutes. $p<0.01$ each) was observed in ionomycin-stimulated SCD neutrophils vs. SCD neutrophils at baseline (*Figures 4B-E*) with no differences observed in baseline ERK and Akt phosphorylation in SCD patients and control volunteers (Figure 4B-E). Treatment of SCD neutrophils with $AnxA1_{Ac2-26}$ showed an 85% reduction in ionomycin-induced ERK phosphorylation at 15 minutes ($p<0.001$ vs. ionomycin-stimulated control neutrophils at 15 minutes) and approximately 75% reduction in ionomycin-induced Akt phosphorylation (30 and 60 minutes. $p<0.01$ at each time point) compared to ionomycin-stimulated control neutrophils (*Figures 4B-E*). Furthermore, as with $AnxA1_{Ac2-26}$ treatment, administration of Akt or Erk inhibitors reduced ionomycin-induced $H3Cit^{+}$SCD neutrophils (*Figure 4F*). Interestingly, in the presence of Z-DEVD-FMK (caspase-3 inhibitor), $AnxA1_{Ac2-26}$ no longer reduced the percentage of $H3Cit^{+}$ neutrophils (*Figure 4G*), but treatment with $AnxA1_{Ac2-26}$ alone increased cleaved caspase-3 (*Figures 4H+I*). Collectively, these data suggest a potential molecular mechanism by which $AnxA1_{Ac2-26}$ can act as a pharmacological switch between the NETosis-apoptosis axis in SCD.

Discussion

Using pharmacological and genetic approaches, coupled with murine and clinical samples we discovered *several key conceptual and novel findings* that we believe advances knowledge and understanding in the field of SCD, thrombo-inflammation and Resolution Biology. Specifically, we found: i) neutrophils from SCD, a known model of thrombo-inflammation, play a major role in cerebral thrombosis; ii) targeting the Fpr2/ALX (a key receptor of inflammation resolution) mitigates these effects; iii) pro-resolving, anti-inflammatory mediator $AnxA1_{Ac2-26}$ reduces $H3Cit^{+}$ rich NET production, transforming neutrophil phenotype from pro-NETotic to pro-apoptotic thereby driving thrombo-inflammation resolution in SCD (*Figure 5*).

Using an experimental model of thrombo-inflammation, i.e. the Townes (STM) mouse (which recapitulates many clinical manifestations of SCD), we revealed heightened cerebral thrombotic responses. By exploiting the Fpr-pathway as a therapeutic target, administration of $AnxA1_{Ac2-26}$ resulted in blood flow prolongation in the cerebral microcirculation of STM (no change was observed in circulating neutrophil counts [*Supplementary Table 1*]) with effects being equally robust in both cerebral venules and arterioles (despite clear differences between vessel types, e.g. shear rates and leukocyte-endothelial interactions).[34] These results demonstrate the potent activity and versatility of the $AnxA1_{Ac2-26}$ to mitigate SCD associated thrombo-inflammation.

Three FPRs exist in the humans: FPR1, FPR2/lipoxin A_4 (Fpr2/ALX, orthologue in the mouse) and FPR3 [13]. Co-administration of $AnxA1_{Ac2-26}$ with Fpr pan-antagonist Boc2 elicited an abrogation of the peptide's protective responses. Moreover, this effect was consistent when the Fpr2/ALX-specific antagonist WRW4 was used, confirming not only the involvement of the Fpr-

family in mediating the effects of $AnxA1_{Ac2-26}$, but more specifically Fpr2/ALX. These findings are the first to show the protective effects of $AnxA1_{Ac2-26}$ in cerebral thrombosis, demonstrating that $AnxA1_{Ac2-26}$ not only possesses anti-inflammatory capabilities (e.g. attenuation of leukocyte-platelet responses post stroke, reduction of lipopolysaccharide-induced leukocyte adhesion and migration), but holds anti-thrombotic capabilities, making it a promising therapeutic candidate for promoting resolution in the context of thrombo-inflammatory diseases such as SCD.

Under normal physiological conditions, the host produces an adequate resolution response to inflammation and coagulation, characterized by specific immunoresolvents that induce clearance mechanisms e.g. apoptosis and efferocytosis.[10,16,35] However, if the resolution process is defective, as observed in chronic thrombo-inflammatory states,[11,36] then this results in the reduction/altered production of pro-resolving mediators as demonstrated by ~~e.g.~~ our SCD patients ~~presented with~~ having reduced circulating AnxA1 plasma levels versus control volunteers. ~~(*Supplementary Figure 3A+B*).~~ We speculate that this chronic deficit contributes to a non-resolving state of inflammation, as previously observed.[16] Interestingly, AnxA1 plasma levels in STM mice were similar to control mice.[11] Although AnxA1 distribution is similar between human and murine neutrophils,[37] differences in AnxA1 levels between species could be due to variances in neutrophil populations (e.g. human neutrophils constitute 65-75% of all peripheral blood leukocytes, unlike in the mouse, where ~10-25% of all leukocytes are neutrophils). Additionally, it was recently shown that resolvin (Rv)D1 (another endogenous pro-resolving mediator) levels were similar between SCD mice vs. controls. However lower levels of RvD1 were detected in spleens (a known target of acute vasoocclusive crises) of SCD mice vs. controls under normoxia or when exposed to hypoxia/reoxygenation,[11] suggesting like AnxA1, other

endogenous mediators may play important roles in thrombo-inflammation resolution. As observed in the clinic, STM mice presented with neutrophilia (*Supplementary Table 2*) and exhibited profound protection against microvascular thrombosis when made neutropenic. Furthermore, upon ~~adoptive~~ transfer of donor STM neutrophils into recipient controls, animals displayed a phenotype similar to that of the full STM mouse, highlighting a key role that circulating STM neutrophils play in mediating cerebral thrombosis, which may translate to the clinical setting.

NET release results in circulating cf-DNA production, which is significantly increased in thrombo-inflammatory diseases, with detrimental effects.[38] Here we discovered increased cfDNA in STM plasma was complexed with NE, which $AnxA1_{Ac2-26}$ was able to reduce, possibly by blocking the attachment of elastase to chromatin in the neutrophil. *In-vivo*, DNase I (main factor regulating elimination of the cfDNA) heightened blood flow cessation times in STM mice, supporting the important role that NETs play in a thrombo-inflammatory environment. Our results suggest a distinctive NETotic phenotype exists in STM and $AnxA1_{Ac2-26}$ specifically targets and reduces excessive NETosis in this thrombo-inflammatory model.

Different studies on thrombosis as well as other inflammatory models including SCD have shown the importance of NET-associated citrullinated histone H3.[29,30] Additionally, histones comprise 65% of the total protein content in neutrophils and extracellular histones (which are the backbone of NETs) are known to participate in immunothrombosis due to their pro-thrombotic,[39] pro-inflammatory and cytotoxic effects.[40] We demonstrated herein that $AnxA1_{Ac2-26}$ was able to significantly modify both the pro-thrombotic STM- and the SCD-NET phenotype by suppressing

the production of citrullinated histone-rich NETs in an AnxA1/FPR2/ALX dependent mechanism. Additionally, the extracellular DNA production was not affected by $AnxA1_{Ac2-26}$ suggesting that the peptide does not reduce physiological netosis.[17,41] These results are in accordance with our *in-vivo* data and may help to further explain the anti-thrombotic action of $AnxA1_{Ac2-26}$. Although we observed some differences in findings from murine neutrophils vs. those with human neutrophils (e.g. NETs released from mice are more compact than those observed from humans),[42] $AnxA1_{Ac2-26}$ was still effective at specifically reducing histone-rich NET production. Interestingly, the effects of $AnxA1_{Ac2-26}$ on $H3cit^{+}$ neutrophils were only observed in STM and SCD neutrophils and not control neutrophils, suggesting that $AnxA1_{Ac2-26}$ spares physiological NETosis. Furthermore, our data shows that once $AnxA1_{Ac2-26}$ is involved in regulating NET formation, it does not affect other neutrophil functions such as MPO release, chemotaxis or cytokine (IL-1β) production but promotes resolution by changing the SCD neutrophil phenotype from a pro-NETotic to pro-apoptotic phenotype.

There is paucity in studies on the cellular mechanisms by which AnxA1/FPR2/ALX can be channelled as an anti-inflammatory/pro-resolving pathway. We further discovered that SCD neutrophils displayed increased ERK and Akt (the main driver of NADPH-independent NET production)[41,43] activation, ensuing in extensive histone citrullination as indicated by NET-based assays.[41] $AnxA1_{Ac2-26}$ inhibited ERK and Akt activity in SCD neutrophils, with inhibitors of ERK and Akt suppressing $H3Cit^{+}$ neutrophil production to similar levels as those with $AnxA1_{Ac2-26}$ treatment. These data demonstrate a molecular mechanism by which $AnxA1_{Ac2-26}$ is able to switch off the pro-thrombotic $H3Cit^{+}$ NETotic drive and enhance the resolution.[35] More importantly $AnxA1_{Ac2-26}$ suppression of ERK and Akt phosphorylation was absent in control

neutrophils. These disparate effects of $AnxA1_{Ac2-26}$ on SCD vs. control phenotype may point towards the configurational plasticity of the FPR signalling axis in response to biased agonism.[44] We also discovered $AnxA1_{Ac2-26}$ to activate cleaved caspase-3 in SCD neutrophils, an effect that was abrogated in the presence of the specific caspase-3 inhibitor (Z-DEVD-FMK). Interestingly, these effects of $AnxA1_{Ac2-26}$ on neutrophil apoptosis are also observed in an acute inflammation model of LPS-induced pleurisy.[45] Taken together, our data provide a previously unknown phenomenon regarding the ability of $AnxA1_{Ac2-26}$ to act as a natural homeostatic clearance ligand during an ensuing thrombotic process.

In summary, our study provides substantial evidence that targeting the AnxA1-FPR2/ALX pathway may provide a viable strategy for the management of thrombotic complications associated with SCD. More specifically, from an intravascular perspective targeting SCD neutrophils via AnxA1-FPR2/ALX pathway reduces $H3Cit^{+}$ NETotic drive which plays a key role (directly or indirectly) in preventing the activation of various downstream processes including platelet aggregation, thereby enabling and promoting resolution. These unique findings may provide impetus to the drug discovery programs based on Resolution Biologics in the management for not only SCD, but also other disease states with an underlying thrombo-inflammatory phenotype.

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Authorship Contributions

J.A. performed experiments, analyzed the data and wrote the manuscript; J.A., E.Y.S., S.A.V., Z.A.Y. and G.K. performed experiments and analyzed the data. J.A., E.Y.S., S.A.V., A.W.O., R.P., R.P.H., D.N.G., P.K, and F.N.E.G. wrote the manuscript. A.W.O., and F.N.E.G. provided reagents, designed and analyzed data. F.N.E.G. was responsible for study supervision.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Figure Legends

Figure 1. AnxA1$_{Ac2-26}$ rescues enhanced cerebral thrombus formation

STM and control mice were subjected to light/dye-induced thrombosis with intravenous infusion of 10mg/kg 5% FITC-dextran followed by photoactivation of cerebral microvessels. (A-D) Images of onset (start of platelet aggregation) and cessation (complete stop of flow for ≥30seconds) of thrombus formation in control and STM mice (scale bar=20µm). Mice were treated with Vehicle (saline), AnxA1$_{Ac2-26}$ (100µg/mouse), AnxA1$_{Ac2-26}$+Boc2 (100µg/mouse+10µg/mouse) or AnxA1$_{Ac2-26}$+WRW4 (100µg/mouse+55µg/mouse), subjected to light/dye-induced thrombosis and time of flow cessation (minutes) was quantified in cerebral (E) arterioles and F) venules. Data shown as mean±SEM (6-7mice/group).$^{***}p<0.001$,$^{****}p<0.0001$ vs. controls.$^{\$\$\$}p<0.001$,$^{\$\$\$\$}p<0.0001$ vs. STM control. $^{\Delta\Delta}p<0.01$,$^{\Delta\Delta\Delta}p<0.001$ vs. STM+AnxA1$_{Ac2-26}$ treated.

Figure 2. Neutrophils contribute to cerebral thrombosis in experimental thrombo-inflammation and exhibit enhanced extracellular DNA activity

Schematic representation of ~~adoptive~~ neutrophil transfer from donor control and STM into neutropenic (anti-neutrophil serum [ANS]) recipient STM followed by light/dye-induced thrombosis (A1-3) time of flow cessation was quantified in cerebral (B) arterioles and (C) venules. (D) Cerebral microvessels were analyzed following DNase (2000U) treatment (n=4-6mice/group).

(E) Plasma levels of circulating Annexin A1 (n=6 each) were also determined in control mice and STM. (F) Neutrophil elastase DNA complex (NE-DNA) levels were determined by ELISA in plasma from saline (vehicle) and AnxA1$_{Ac2-26}$-treated mice (n=12 saline and n=6 AnxA1$_{Ac2-26}$

treated control and STM [Two values for control-AnxA1$_{Ac2-26}$ and one value STM-AnxA1$_{Ac2-26}$ were under detectable levels and not included]). (G) percentage histone H3 (H3Cit^{+}) positive unstimulated (n=10 [one outlier removed]. n=10 STM [one outlier removed]) and ionomycin-stimulated (n=10 control, n=7 STM. [4µM]) neutrophils. Data shown as mean±SEM from independent experiments. $^{*}p<0.05$ vs. control mice. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ vs. STM, $^{@@}p<0.01$ vs. unstimulated STM neutrophils and $^{\$}p<0.05$ vs. stimulated STM neutrophils.

Figure 3. SCD-associated enhanced H3Cit^{+} neutrophils can be inhibited by AnxA1$_{Ac2-26}$

(A) Schematic representation of neutrophil isolation and neutrophil extracellular trap (NET) analysis. (B) Representative images of NETs (H3Cit [green/Alexa Fluor 488], neutrophil elastase [NE] [red/Alexa Fluor 568], and nucleus [DAPI]. Scale bars: 100µM and 10µM [inset]). (C) Percentage of NETs hypercitrullinated at histone H3 (H3Cit^{+}) quantified from unstimulated and ionomycin-stimulated neutrophils from control volunteers (unstimulated [n=10, one outlier removed] and stimulated [n=10, one outlier removed]), and SCD patients (unstimulated [n=10] and stimulated [n=14]). (D) Plasma levels of circulating Annexin A1 (n=5, 6 respectively) was determined in control volunteers and SCD patients. Statistical significance was determined using unpaired t test and presented as $*p<0.05$ vs. control volunteers. (E) Percentage of H3Cit^{+} ionomycin-stimulated neutrophils from control volunteers (n=8 vehicle and n=9 AnxA1$_{Ac2-26}$ pre-treatment) and SCD patients (n=14 vehicle, n=9 AnxA1$_{Ac2-26}$, n=10 AnxA1$_{Ac2-26}$+Boc-2 and n=9 AnxA1$_{Ac2-26}$+WRW4). Data expressed as mean±SEM from independent experiments. $^{*}p<0.05$,$^{**}p<0.01$ vs. control unstimulated neutrophils.$^{\#\#\#\#}p<0.0001$ vs. SCD unstimulated neutrophils.$^{\$\$\$\$}p<0.001$ vs. ionomycin-stimulated SCD neutrophils. $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$ vs. SCD+AnxA1$_{Ac2-26}$ treated SCD neutrophils. $^{\varphi\varphi\varphi}p<0.001$ vs. stimulated control neutrophils.

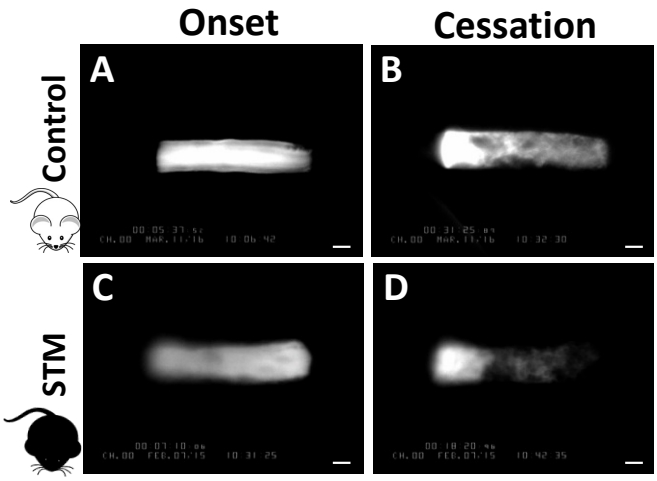
Figure 4. $AnxA1_{Ac2-26}$ dampens ERK and Akt activation in neutrophils isolated from SCD patients and activates cleaved caspase-3.

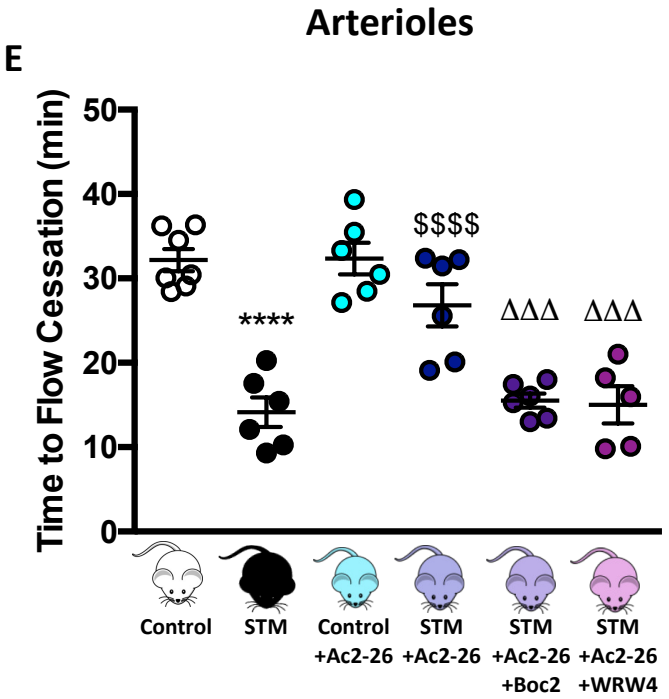
A) Schematic representation of sample preparation for western blotting. Representative western blots of neutrophils from control volunteers and SCD patients for (B) ERK activation and (C) Akt activation after $AnxA1_{Ac2-26}$ treatment and ionomycin stimulation. Densitometric analysis of (D) p-ERK/total ERK (n=5) and (E) p-Akt/total Akt (n=4. One outlier [defined as ≥two standard deviations] removed from control ionomycin+$AnxA1_{Ac2-26}$ [30minutes]). (F+G) Percentage of $H3Cit^{+}$ SCD-neutrophils following ionomycin stimulation (4µM, 3hours), with/without pre-treatment with $AnxA1_{Ac2-26}$ (30µM, 15minutes) and Akt (10µM, 30minutes), ERK (10µM, 60minutes) or caspase-3 (Z-DEVD-FMK. 20µM, 45minutes) inhibitors (n=5 each group). (H) Representative immunofluorescence images of cleaved caspase-3 staining from SCD-neutrophils with/without $AnxA1_{Ac2-26}$ (30µM) treatment. (I) Percentage of cleaved caspase-3^{+} neutrophils from control and SCD patients with/without $AnxA1_{Ac2-26}$ treatment (n=5). Data expressed as mean±SEM from independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. unstimulated control neutrophils, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. unstimulated SCD neutrophils, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$, $^{\$\$\$\$}p<0.0001$ vs. ionomycin stimulated SCD neutrophils at the corresponding time points. $^{\Delta\Delta}p<0.01$ vs. $AnxA1_{Ac2-26}$ pre-treated ionomycin stimulated SCD neutrophils.

Figure 5. Schematic of proposed mechanisms.

Our data show (A) SCD neutrophils produce increased NETs, which are exacerbated upon stimulation (e.g. ionomycin) leading to increased phosphorylation of NET specific kinases (ERK

and Akt), which has been shown to result in histone citrullination and inhibition of apoptosis via upregulation of anti-apoptotic proteins e.g. Mcl-1[33] (**Pro-thrombotic state**). Peptidylarginine deiminase 4 (PAD4) forms a complex with intracellular calcium to catalyze histone citrullination[31]. NET stimuli activate PKC, PLC and PI3K,[33] which in-turn activate ERK and AKT, resulting in Calcium-PAD4 complex formation, which catalyzes histone citrullination. We found (B) $AnxA1_{Ac2-26}$ interacts with Fpr2/ALX suppressing ERK and Akt phosphorylation, preventing histone citrullination and enabling apoptosis by activating caspase-3 (**Pro-resolving state**). *Abbreviations*: ERK, extracellular signal-regulated kinases; Akt, protein kinase B; Mcl-1, myeloid cell leukemia protein-1; PKC, protein kinase C; PLC, Phospholipase C; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase.


Onset
Cessation
Control
STM
A
B
C
D


E
Arterioles
Time to Flow Cessation (min)
50
40
30
20
10
0
****
$$$$
ΔΔΔ
ΔΔΔ
Control
STM
Control +Ac2-26
STM +Ac2-26
STM +Ac2-26 +Boc2
STM +Ac2-26 +WRW4

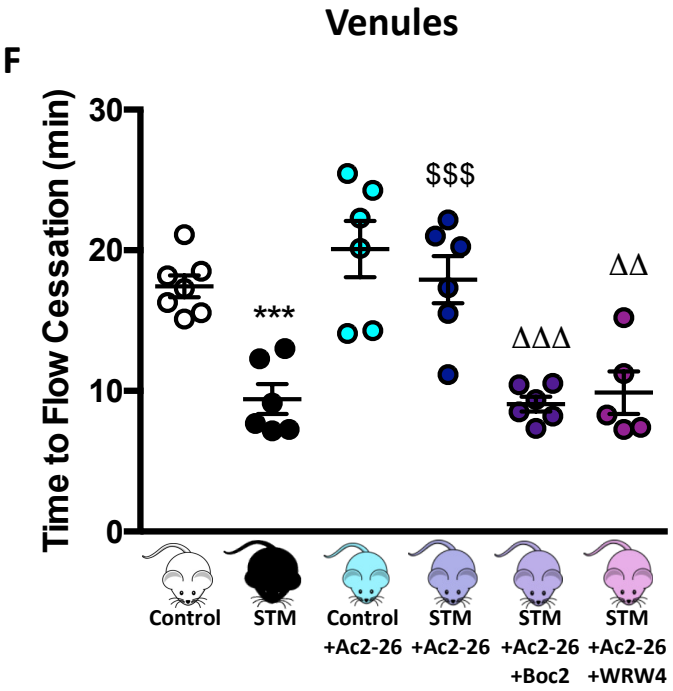

F
Venules
Time to Flow Cessation (min)
30
20
10
0
***
$$$
ΔΔΔ
ΔΔ
Control
STM
Control +Ac2-26
STM +Ac2-26
STM +Ac2-26 +Boc2
STM +Ac2-26 +WRW4

Figure 1

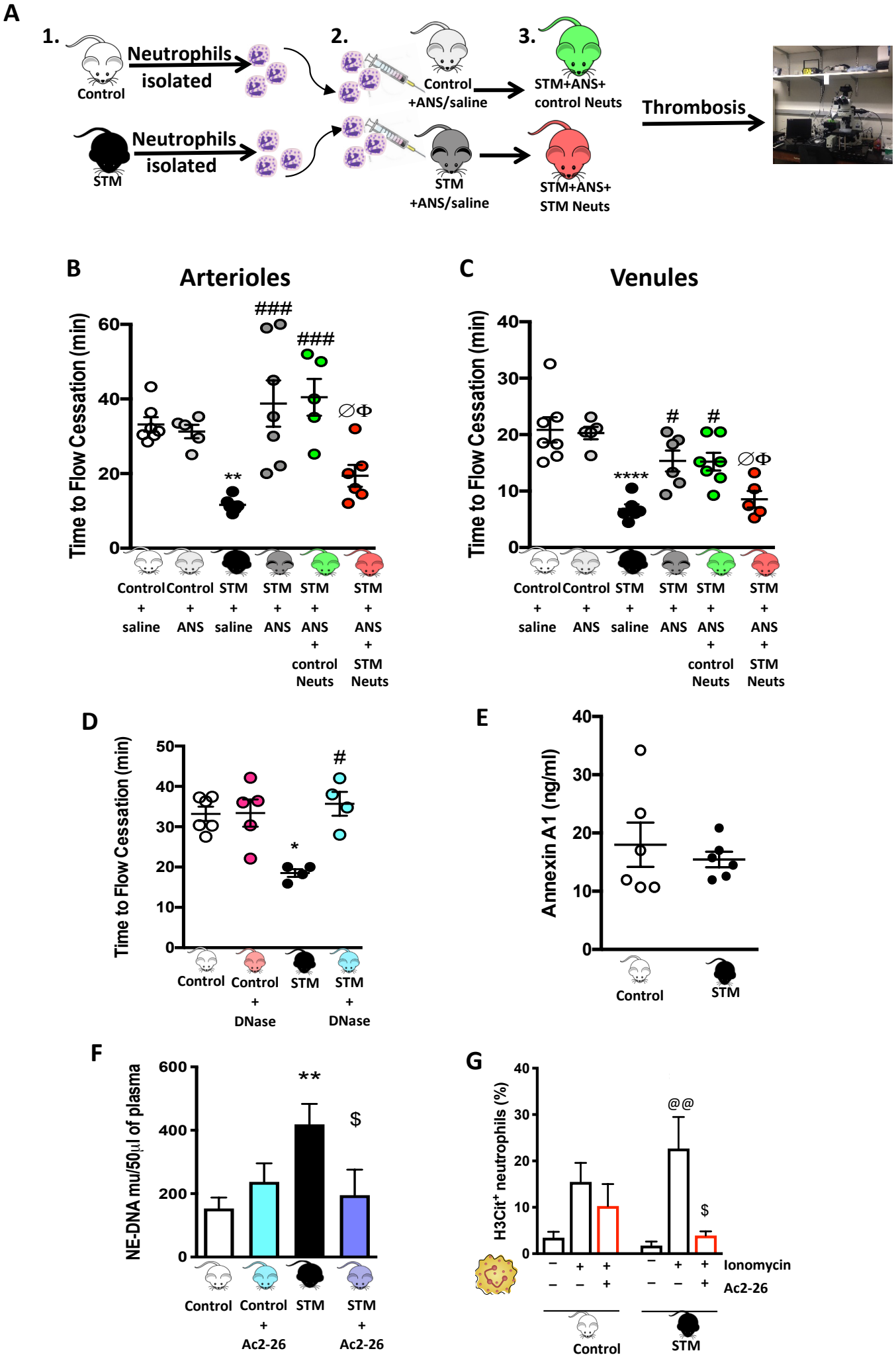

A
1.
Control
Neutrophils isolated
STM
Neutrophils isolated
2.
Control +ANS/saline
STM +ANS/saline
3.
STM+ANS+ control Neuts
STM+ANS+ STM Neuts
Thrombosis
B
Arterioles
Time to Flow Cessation (min)
Control + saline
Control + ANS
STM + saline
STM + ANS
STM + ANS + control Neuts
STM + ANS + STM Neuts
C
Venules
Time to Flow Cessation (min)
D
Time to Flow Cessation (min)
Control
Control + DNase
STM
STM + DNase
E
Annexin A1 (ng/ml)
Control
STM
F
NE-DNA mu/50µl of plasma
Control
Control + Ac2-26
STM
STM + Ac2-26
G
H3Cit+ neutrophils (%)
Ionomycin
Ac2-26
Control
STM

Figure 2

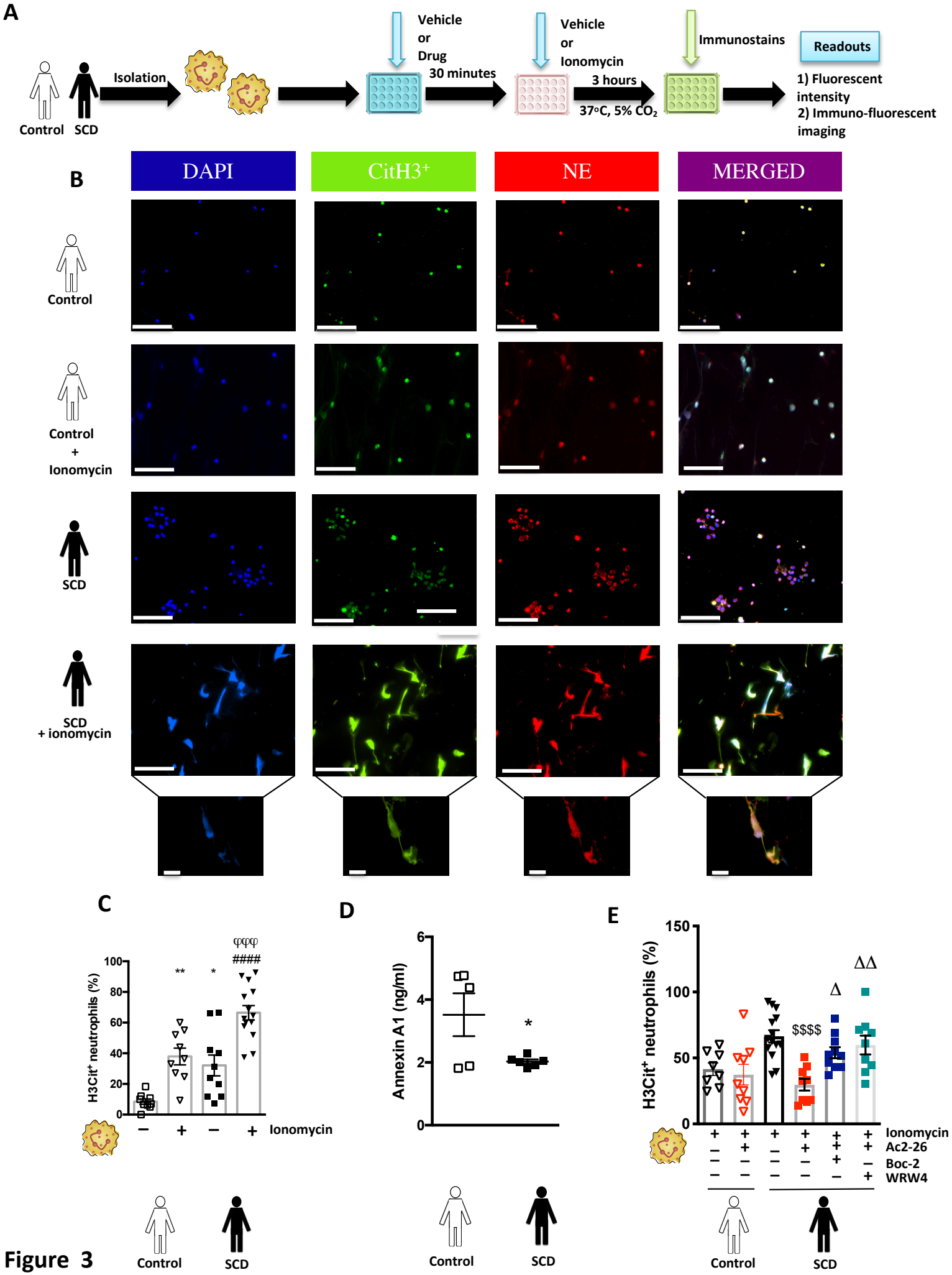

A
Control
SCD
Isolation
Vehicle
or
Drug
30 minutes
Vehicle
or
Ionomycin
3 hours
37°C, 5% CO2
Immunostains
Readouts
1) Fluorescent intensity
2) Immuno-fluorescent imaging
B
DAPI
CitH3+
NE
MERGED
Control
Control + Ionomycin
SCD
SCD + ionomycin
C
H3Cit+ neutrophils (%)
Ionomycin
D
Annexin A1 (ng/ml)
E
H3Cit+ neutrophils (%)
Ionomycin
Ac2-26
Boc-2
WRW4
Control
SCD

Figure 3

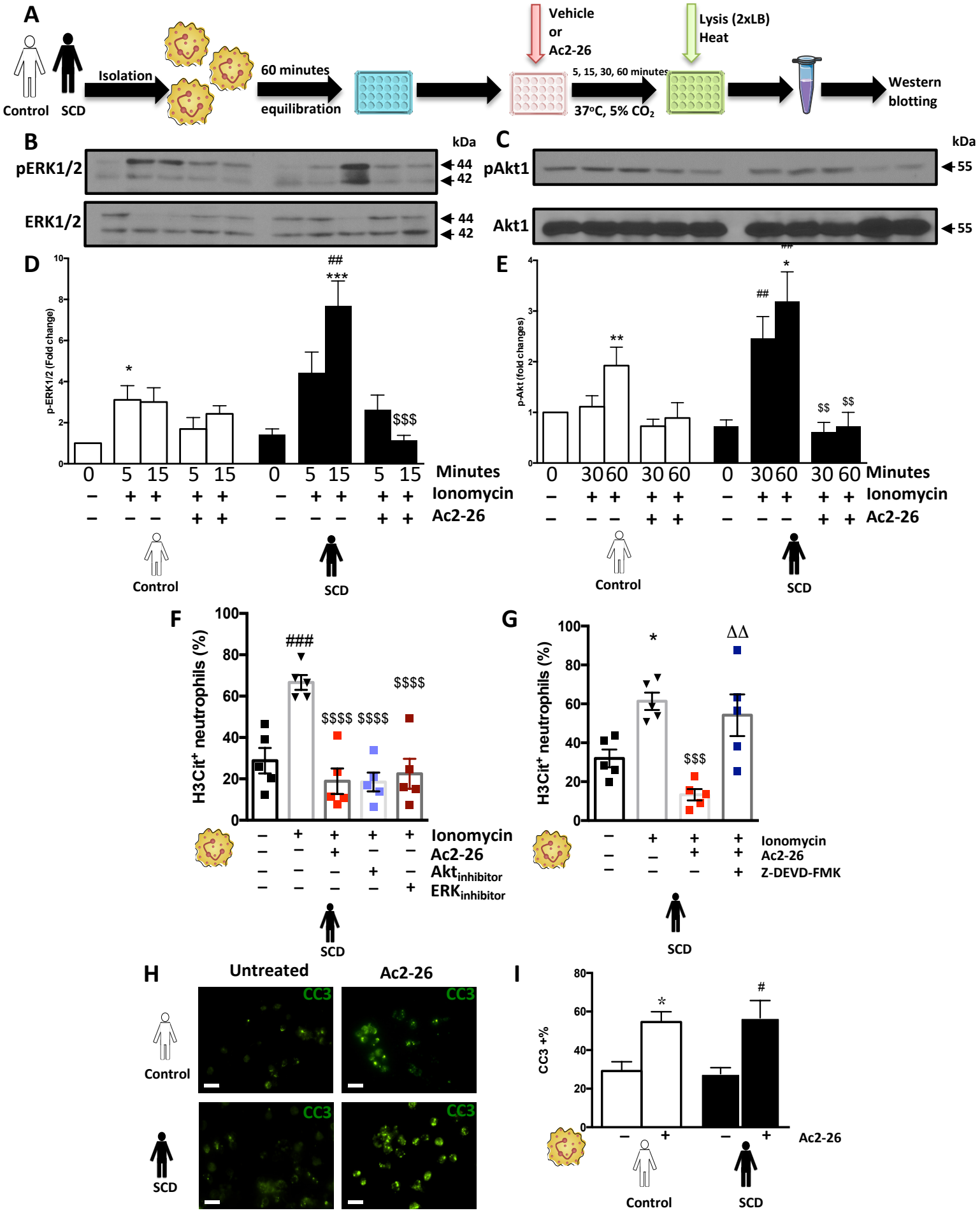

A
Control
SCD
Isolation
60 minutes
equilibration
Vehicle
or
Ac2-26
5, 15, 30, 60 minutes
37°C, 5% CO2
Lysis (2xLB)
Heat
Western
blotting
B
kDa
pERK1/2
ERK1/2
44
42
C
pAkt1
Akt1
55
D
p-ERK1/2 (Fold change)
Minutes
Ionomycin
Ac2-26
Control
SCD
E
p-Akt (fold changes)
F
H3Cit+ neutrophils (%)
Ionomycin
Ac2-26
Akt inhibitor
ERK inhibitor
G
Z-DEVD-FMK
H
Untreated
Ac2-26
CC3
I
CC3 +%

Figure 4

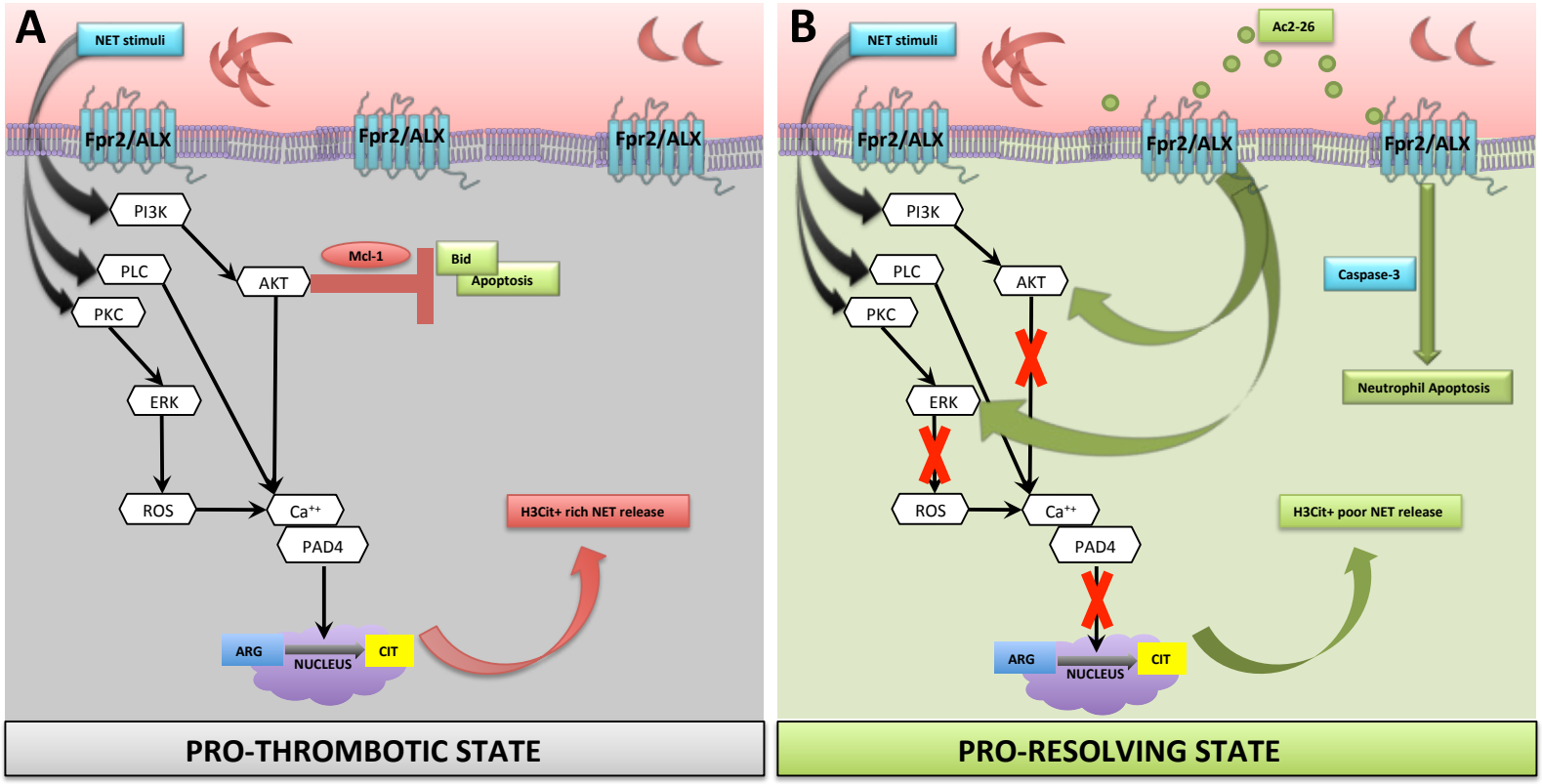

A
NET stimuli
Fpr2/ALX
Fpr2/ALX
Fpr2/ALX
PI3K
PLC
PKC
AKT
Mcl-1
Bid
Apoptosis
ERK
ROS
Ca++
PAD4
H3Cit+ rich NET release
ARG
NUCLEUS
CIT
PRO-THROMBOTIC STATE
B
NET stimuli
Ac2-26
Fpr2/ALX
Fpr2/ALX
Fpr2/ALX
PI3K
PLC
PKC
AKT
Caspase-3
Neutrophil Apoptosis
ERK
ROS
Ca++
PAD4
H3Cit+ poor NET release
ARG
NUCLEUS
CIT
PRO-RESOLVING STATE

Figure 5