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Nanomedicine platform for targeting activated neutrophils and neutrophil–platelet complexes using an α_1-antitrypsin-derived peptide motif

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M.A.C., D.B., E.A.A., M.T.N., T.S.K., K.B.N., C.M., S.d.M., M.D.N., A.S.G. and E.X.S. conceptualized and planned the experiments. M.A.C., D.B., E.A.A., J.A., S.R., N.A.M., N.D.v.K., K.L.B., S.H., K. Hageman, K.M., M.S., H.L., A.B., E.M.L., K. Hart, A.G., M.d.l.F. and E.X.S. performed the experiments. M.A.C., S.d.M., A.S.G. and E.X.S. prepared the figures. M.A.C. and E.X.S. wrote the manuscript, and all authors reviewed and edited the manuscript before submission.

Competing interests

A.S.G. is a coinventor on issued patent US 9107845 (Synthetic Platelets) that is licensed from Case Western Reserve University to Haima Therapeutics. A.S.G. is a cofounder and equity stakeholder of Haima Therapeutics. The patent is on the design of a heteromultivalent NP system that can mimic the haemostatic functions of a platelet. A.S.G. is also a coinventor on issued patent US 9107963 (Heteromultivalent Nanoparticle Compositions). The patent is on the design of heteromultivalently decorated NPs for clot targeting. Although the specific NP systems described in these two patents have no direct relevance to any specific aspect of the manuscript, the context of 'heteromultivalent NP design' is a central aspect of the NT-NP and PNT-NP systems described in the manuscript. M.D.N. serves on the scientific advisory board of Haima Therapeutics and holds equity stake. E.X.S. is coinventor of intellectual property that has been licensed by Case Western Reserve University to XaTek and receives royalties. The patent PCT/US2017/013797 is on dielectric spectroscopy for whole blood assessment of haemostasis. This patent bears no relevance to any of the work presented in the manuscript. C.M. has been a speaker for Shire-Takeda. C.M. and S.d.M. are cofounders of TargED BV, a biotech spinout company of University Medical Center Utrecht (based upon the WO2019185723 A1 patent). C.M. and S.d.M. participate in revenue sharing as inventors through the commercialization arm of the University Medical Center Utrecht. The remaining authors declare no competing interests.

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Abstract

Targeted drug delivery to disease-associated activated neutrophils can provide novel therapeutic opportunities while avoiding systemic effects on immune functions. We created a nanomedicine platform that uniquely utilizes an α_1-antitrypsin-derived peptide to confer binding specificity to neutrophil elastase on activated neutrophils. Surface decoration with this peptide enabled specific anchorage of nanoparticles to activated neutrophils and platelet–neutrophil aggregates, in vitro and in vivo. Nanoparticle delivery of a model drug, hydroxychloroquine, demonstrated significant reduction of neutrophil activities in vitro and a therapeutic effect on murine venous thrombosis in vivo. This innovative approach of cell-specific and activation-state-specific targeting can be applied to several neutrophil-driven pathologies.

Neutrophils are the first line of host defence, facilitating the containment and destruction of microbial pathogens by releasing granular enzymes, reactive oxygen species and neutrophil extracellular traps (NETs)[1]. While neutrophils are an integral component of the innate immune response, recent evidence supports the unrestricted recruitment and function of activated neutrophils can prolong inflammation and contribute to the development of pathologic conditions such as vascular thrombosis, tumour progression, autoimmune diseases and chronic, non-healing wounds[2]. Therefore, substantial research endeavours have emerged in recent years to develop strategies that modulate neutrophil functions as a treatment for neutrophil-driven pathologies[3–5]. However, studies have revealed considerable challenges with these approaches, primarily stemming from the inhibition of essential

neutrophil functions, the limited half-life of therapeutic agents and potential off-target effects associated with systemic delivery[6–8]. Therefore, targeted delivery of therapeutic agents specifically to disease-associated, activated, pro-inflammatory neutrophils can ensure an effective drug half-life, localized action and minimal side effects in treating neutrophil-driven pathologies.

The ‘nanomedicine’ approach of targeted drug delivery can provide such a strategy, whereby therapeutic agents can be packaged within nanoparticles (NPs), and the particle surface can be decorated with specific ligands that enable selective binding to disease-associated cells and tissues[9–11]. This approach has become highly promising in the targeted delivery of therapeutics in cancer[11,12] and, to some extent, cardiovascular[13] pathologies. Efforts have focused on developing neutrophil-targeted NPs, but the current targeting approaches are not unique or specific to activated neutrophils[14–16]. This presents a remarkable opportunity to develop a neutrophil-specific nanomedicine platform.

Ligand design and NP assembly

To render NP binding specifically to activated neutrophils and platelets, we first identified binding targets on the surface of these cells. For neutrophils, we focused on neutrophil elastase (NE) because (1) it is exposed on the neutrophil surface only upon cell activation and (2) in contrast with other membrane-bound proteins (for example, integrins), NE is exclusively present on neutrophils but not on other leucocyte subsets. Although NE is secreted from neutrophils, any free-circulating NE is rapidly and completely neutralized by anti-proteases (Fig. 1a)[17]. However, at the site of secretion, the millimolar concentration of NE outcompetes the micromolar concentration of human α1-antitrypsin[17,18] (AAT; Fig. 1a). For platelet targeting, we focused on P-selectin since this translocates to the plasma membrane only upon platelet activation[19].

To target NE on activated neutrophils, we designed a 14 amino acid peptide (CGEAIPMSIPPEVK) termed NE-binding peptide (NEBP) that binds to NE (Fig. 1b,c)[20,21]. This peptide sequence was derived from the reactive centre loop of AAT, a serpin that physiologically binds to NE (Fig. 1b)[22]. We preserved the methionine at the P1 position and added a cysteine at the P7 position for conjugation of NEBP to lipids for NP construction (Fig. 1f). High-performance liquid chromatography showed no discernible change in the mass spectra of NEBP following incubation with human or murine NE (hNE or mNE; Fig. 1d), supporting that NE does not cleave NEBP. To study the binding of NEBP with mNE, surface plasmon resonance was performed, which showed that NEBP potently bound to mNE with an association rate constant k_{on} of 5.2×10^3 M^{-1} s^{-1}, a dissociation rate constant k_{off} of 8.31×10^{-6} s^{-1} and an equilibrium dissociation constant K_D of 1.6 nM (Fig. 1e). Binding between NEBP and free hNE was first characterized with microscale thermophoresis (Supplementary Fig. 1a). Based on microscale thermophoresis measurements, a binding constant of 2.077 ± 1.353 μM was determined for NEBP and hNE, and 0.1375 ± 0.0014 μM for AAT and hNE (Supplementary Fig. 1a). An enzyme-sensitive probe, *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin, or NMeOSuc-AAPV-AMC (AMC-substrate), was also used to measure the interaction of NEBP with hNE (detection limit for hNE, 0.01 nM). Rising concentrations of AAT (10–120

nM) abolished hNE activity (Supplementary Fig. 1b). NEBP (60–1,000 nM) similarly led to a concentration-dependent decrease in hNE activity (Supplementary Fig. 1b). The second-order rate constant (k_2) of inhibition of NE by AAT was (7.6 ± 1.69) × 10^6 M^{-1} s^{-1}, whereas for NEBP, k_2 was (2.5 ± 1.04) × 10^5 M^{-1} s^{-1} (mean ± s.e.m.). Since hNE is predominantly active on the surface of activated neutrophils[23], we next examined if NEBP can effectively bind to membrane-exposed hNE. We employed a specific small-molecule ratiometric NE reporter based on Forster resonance energy transfer, termed NEmo-2 (detection sensitivity for hNE, 0.02 nM)[23]. *N*-formylmethionine-leucyl-phenylalanine (fMLP) activation of neutrophils led to binding of NEmo-2 to membrane-exposed hNE and resulted in Forster resonance energy transfer reporter cleavage where the donor channel mean fluorescent intensity increased over time, and the acceptor channel mean fluorescent intensity decreased or remained constant (Supplementary Fig. 1c). Incubating activated neutrophils with AAT or NEBP led to a pronounced decrease in Forster resonance energy transfer reporter cleavage compared to fMLP-activated neutrophils (Supplementary Fig. 1c,d). As a control, cultured HEK-293 cells (which do not express NE) were incubated with NEmo-2 and showed no change in donor/acceptor ratio (Supplementary Fig. 1c). Since NEmo reporters provide ~34-fold specificity for hNE over proteinase 3 (PR3; membrane bound) and NEmo-2 does not capture binding to cathepsin G (secreted)[23], we assessed if NEBP interacts with these proteases. Coincubating PR3 or cathepsin G with rising concentrations of NEBP did not lead to a decrease in AMC-substrate fluorescence or succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide absorbance, respectively (Supplementary Fig. 1e,f), indicating that NEBP does not bind to either protease. Peptide GEAIPMSIPPEVK (termed sNEBP), which lacks the terminal cysteine at the P7 position of NEBP, was similarly found to not interact with PR3 or cathepsin G (Supplementary Fig. 1e,f).

It has been recognized that AAT has the ability to interact with multiple serine protease targets, among them components of the fibrinolytic system[24]. To investigate if NEBP binds to and inhibits non-neutrophil-derived serine proteases like plasmin or tissue plasminogen activator (tPA), we measured plasmin activity, plasmin generation and clot lysis times, with and without NEBP. These studies showed that in contrast to AAT, which abolished all residual plasmin activity, NEBP had no effect when compared to the vehicle (Supplementary Fig. 2a,b). Similarly, NEBP did not affect tPA-induced plasminogen activation (Supplementary Fig. 2c,d) or plasma clot lysis times (Supplementary Fig. 2e–g).

To target P-selectin on activated platelets, we selected the phage-display-generated peptide sequence DAEWVDVS, which has high affinity and specificity to P-selectin (IC_{50} = 6 μM) compared to native sialyl Lewis acid ligands[25]. A cysteine was added to this sequence to enable conjugation to lipids for liposomal NP assembly, and this sequence was termed P-selectin binding peptide (PBP; Fig. 1f). We previously demonstrated the utilization of this peptide in NP targeting to ADP-activated platelets[26].

We carried out two combinations of NP surface decoration: (1) we combined two neutrophil-specific ligands, NEBP and an antigen-binding fragment (Fab) derived from anti-Ly6G antibody (clone 1A8)[27], to form heteromultivalent neutrophil-targeted NPs (NT-NPs; Fig. 1f); and (2) we combined NEBP with PBP to create heteromultivalent NPs that simultaneously bind to activated neutrophil–platelet aggregates (henceforth called

platelet–neutrophil-targeted NPs, or PNT-NPs; Fig. 1f). Full details on NP assembly[28–30] are provided in the Methods. Mass spectrometry confirmed the successful conjugation of NEBP and PBP peptides and Ly6G–Fab fragment to lipids (Supplementary Fig. 3a–c). NPs were 100–200 nm in diameter, measured by dynamic light scattering and cryo transmission electron microscopy (Fig. 1g,h). Lastly, kinetic surface plasmon resonance experiments showed that assembled NT-NPs bound to NE with a k_{on} of 9.7×10^2 M^{-1} s^{-1}, k_{off} of 2.94 μM s^{-1} and K_D of 20 nM (Fig. 1i).

NT-NPs selectively bind to activated neutrophils in vitro and in vivo

Ly6G expression, although exclusively found on murine neutrophils, is not specific to activated cells, and high concentrations of Ly6G-specific antibodies can deplete neutrophils[27]. Therefore, we used a considerably higher mole percent of NEBP (2.5 mol%) over Ly6G–Fab (0.1 mol%) for NT-NP decoration (Fig. 2a) and measured the cell viability of murine wild-type neutrophils in the presence of NPs. Neutrophil viability was preserved by >90% when cells were incubated with 3×10^9 ml^{-1} NT-NP (1:100 dilution; Fig. 2b). Based on these results, we utilized 3×10^9 ml^{-1} NPs for all in vitro and in vivo studies with NT-NP.

To assess NP binding specificity towards activated neutrophils in vitro, wild-type neutrophils were incubated with untargeted (U) or targeted (NT) NPs, in the absence or presence of fMLP. Immunofluorescence images showed that U-NPs (rhodamine B (RhB)-labelled only) did not bind to inactive or activated cells (Fig. 2c (upper two panels) and Fig. 2d; $P = 0.14$). By contrast, NT-NPs bound exclusively to activated neutrophils (lower right panel) but not to inactive cells (Fig. 2c (lower left panel) and Fig. 2d; $P < 0.0001$).

Circulation half-life ($T_{1/2}$), defined as the time where relative residual NP RhB signal is 50%, was determined to be ~8 hours for both U-NPs and NT-NPs (Fig. 2e). To assess the binding specificity of NT-NPs in vivo, systemic inflammation in wild-type mice was induced with intravenous (i.v.) administration of lipopolysaccharide (LPS; 1 mg kg^{-1} body weight), as previously described[31]. Three hours later, mice were injected with RhB-labelled NPs (U-NPs or NT-NPs) and Alexa Fluor-488 anti-Gr-1 antibody. NPs were allowed to circulate for 40 min before mice were perfused with Concanavalin A conjugated to fluorescein isothiocyanate to delineate the retinal microvasculature. Mice injected with phosphate-buffered saline (PBS) and U-NP or NT-NP showed an absence of neutrophil or NP accumulation into retinal capillaries (left upper and left lower panels, Fig. 2f). By contrast, LPS-induced systemic inflammation resulted in robust neutrophil recruitment into the retina (right upper and right lower panels, Fig. 2f). However, NP binding to these activated neutrophils was evident only in mice that had received NT-NPs (right lower panel) and not in mice injected with U-NPs (right upper panel, Fig. 2f). Analysis showed minimal colocalization between activated neutrophils and U-NPs but extensive colocalization between activated neutrophils and NT-NPs ($P < 0.000001$; Fig. 2g,h). We next investigated if decorating NPs exclusively with NEBP would maintain binding selectivity and the retention of NPs on activated neutrophils, while allowing studies to be performed with both murine and human cells. These new NPs, termed NEBP-NP, were synthesized as described above. To test NP binding specificity, we performed in vitro immunofluorescence

and flow cytometry studies. Human neutrophils were incubated with media or activated with various agonists (phorbol myristate acetate (PMA), tumour necrosis factor alpha (TNF-α), LPS, fMLP), and NEBP-NPs or U-NPs were added. NEBP-NPs bound to activated neutrophils and NETs but did not interact with inactive cells ($P = 0.0022$), whereas U-NPs did not colocalize with either inactive or activated neutrophils (Fig. 3a,b). Similarly, flow cytometry showed no significant binding of U-NPs to neutrophils, irrespective of the cell activation state (Fig. 3c). By contrast, the mean fluorescent intensity for RhB was significantly increased when NEBP-NPs were coincubated with activated neutrophils (Supplementary Fig. 4a) but not with inactive cells (Fig. 3c).

Investigations next examined how neutrophils process NEBP-NPs. Live human neutrophils were treated with media or stimulated with 1 μM fMLP and coincubated with U-NPs or NEBP-NPs for 2 hours. On completion of incubation, the plasma membrane, lysosomes and nuclei were fluorescently labelled and analysed with confocal imaging. U-NPs did not localize on the surface of inactive neutrophils, as seen by the lack of colocalization between RhB and plasma membrane stains (Supplementary Fig. 5 and Fig. 4a–c). Similarly, when U-NPs were incubated with fMLP-activated neutrophils, no NP retention or cell uptake was observed. NEBP-NPs coincubated with inactive neutrophils did exhibit minimal NP binding on the plasma membrane, likely due to a degree of neutrophil activation after 2 hours of incubation (Supplementary Fig. 5 and Fig. 4a–c). NEBP-NPs incubated with fMLP-activated cells significantly bound to the neutrophil surface and, in contrast to any other condition, NEBP-NPs were also internalized (Supplementary Fig. 5 and Fig. 4a–c). Composite images revealed partial intracellular trafficking of NEBP-NPs to lysosomes, albeit the largest NP proportion remained bound at the plasma membrane (Supplementary Fig. 5 and Fig. 4a–c).

PNT-NPs effectively target activated platelet–neutrophil aggregates

In recent years, multiple animal and clinical studies have identified that inflammatory processes and deep vein thrombosis (DVT) are intricately linked[32–34]. Therefore, we hypothesized that PNT-NPs can be utilized to target activated platelet–neutrophil complexes (Fig. 5a). Human platelets and neutrophils, activated with 20 nM thrombin and 1 μM fMLP, respectively, were incubated with 3×10^9 ml^{-1} RhB-labelled NPs (U-NP, NEBP-NP, PBP-NP or PNT-NP) and images were used to quantitate RhB, a measure of NP retention on cell aggregates (Fig. 5b). There was minimal RhB fluorescence when U-NPs were incubated with activated platelets and neutrophils (Fig. 5b (middle row panels) and Fig. 5c). By contrast, PNT-NPs were able to significantly bind to activated platelets and neutrophils (Fig. 5b (bottom row panels) and Fig. 5c) compared to U-NPs ($P < 0.001$). Single decoration of NPs with NEBP or PBP each resulted in significantly higher NP targeting to activated platelets and neutrophils compared to U-NP ($P < 0.0001$), but combinatorial decoration with NEBP and PBP synergistically conferred significantly higher binding efficacy of PNT-NPs compared to either NEBP-NPs or PBP-NPs ($P < 0.0001$; Supplementary Fig. 6a,b). In flow cytometry analyses, no significant increase in RhB median fluorescent intensity occurred when inactive neutrophils (IN) and platelets (IP) were coincubated with U-NPs or PNT-NPs (Fig. 5d (upper two panels) and Fig. 5e). By contrast, coincubation of activated cell complexes with PNT-NP (Fig. 5d, lower two panels) resulted in a significant increase in RhB median fluorescent intensity compared to treatment with U-NPs (Fig. 5d,e).

The above results provided the rationale to study whether PNT-NPs can bind to a DVT-relevant thrombotic niche under flow for potential drug delivery. For this, we employed a custom DVT microfluidic model where clot formation is initiated by immobilized tissue factor and supported by the low shear vortical flows within the valve pocket (Fig. 6a)[35]. Time-lapse video microscopy demonstrated significantly increased accumulation of PNT-NPs compared to U-NPs in the thrombus forming within the valve pocket (Fig. 6b). PNT-NP binding to recruited platelets and neutrophils was observed at early time points, and for several time frames, PNT-NPs could be seen 'following' an activated neutrophil or platelet (Supplementary Video 1). By contrast, U-NPs did not adhere at the thrombus site until NET and fibrin formation had occurred (Fig. 6b), indicating that NPs were non-specifically trapped within the thrombotic niche rather than specifically binding to the thrombus (Supplementary Fig. 7a,b and Supplementary Video 2). Soluble P-selectin did not interfere with NP binding to activated platelets36 (Supplementary Fig. 8a,b).

Targeted therapeutic effect of PNT-NP nanomedicine in vivo

For proof-of-concept DVT studies with drug-loaded NPs, we chose to use hydroxychloroquine (HCQ) since (1) we previously showed that intraperitoneal administration of HCQ reduced thrombus burden in murine models of trauma[37]; (2) HCQ is an autophagy inhibitor and can potentially impact neutrophil functions[38]; and (3) there is considerable dose-limiting toxicity with systemic HCQ treatment[39–41]. HCQ was encapsulated within PNT-NPs to yield HCQ_PNT-NPs (Supplementary Fig. 9a,b). When neutrophils were incubated with free HCQ, cell viability fell below 90% at HCQ concentrations equal to or higher than 2×10^3 ng ml^{-1} (Fig. 6c). By contrast, neutrophil incubation with HCQ_PNT-NPs did not result in cell cytotoxicity across all tested concentrations (0–3×10^6 ng ml^{-1}; Fig. 6c). We next investigated if HCQ influences key neutrophil effectors involved in sterile inflammatory and thrombotic processes, among them NE secretion and NET-osis[42]. To allow comparisons between free HCQ and HCQ-loaded PNT-NPs while not affecting neutrophil viability, all in vitro mechanistic studies were performed at a fixed HCQ concentration (350 ng ml^{-1}), which fell well within the range of non-cytotoxic dosing (Fig. 6c). Treatment of fMLP-stimulated neutrophils with unencapsulated HCQ led to a significant reduction in NE activity compared to fMLP without HCQ ($P < 0.0001$). Importantly, treatment of neutrophils with the same concentration of HCQ packaged in PNT-NPs (HCQ_PNT-NPs) resulted in a comparable reduction of NE activity as with free HCQ ($P < 0.0001$ versus fMLP; Fig. 6d). Since NE associates with NETs[32], we studied whether a HCQ-mediated decrease in extracellular NE activity can translate into reduced NET formation. fMLP stimulation led to a rapid increase in citrullinated histone H3 expression, which was significantly reduced by free HCQ ($P < 0.0001$; Supplementary Fig. 4c,d and Fig. 6e). Notably, HCQ_PNT-NPs resulted in the highest reduction in citrullinated histone H3 expression ($P < 0.0001$ versus fMLP; Fig. 6e). For in vivo studies, we established that the circulation half-life of PNT-NPs was similar to that of NT-NPs at ~8 hours (Supplementary Fig. 10a). In terms of systemic safety, i.v.-free HCQ was found to be highly lethal to mice, whereas i.v. HCQ_PNT-NPs had no effect on survival (final HCQ, 350 μg per mouse in both groups; Fig. 6f). For therapeutic studies, we utilized a murine model of DVT in which flow restriction induces thrombosis in the

inferior vena cava (IVC; Fig. 6g)[32]. Saline (no treatment), empty untargeted NPs (U-NPs), HCQ_U-NPs (containing HCQ but no surface decorations) or HCQ_PNT-NPs (codecorated with NEBP + PBP and loaded with HCQ) were i.v. administered 30 minutes prior to surgical induction of IVC ligation, and IVC thrombi were harvested 24 hours later. Lack of NP surface decorations (U-NP) resulted in thrombus weights identical to control wild-type mice, irrespective of HCQ loading (Fig. 6h). Distinctly, PNT-NPs loaded with HCQ led to significantly smaller thrombi compared to every other group (Fig. 6h). Systemic i.v. administered HCQ at a 350 μg dose did not result in significantly lower thrombus weights compared to no treatment ($P = 0.1$; Supplementary Fig. 10b), likely due to the low number of surviving animals.

To establish the selective contribution of neutrophil targeting and platelet targeting to the therapeutic effect of thrombus weight reduction, HCQ was encapsulated in NPs bearing NEBP (HCQ_NEBP-NPs) or PBP (HCQ_PBP-NPs) decorations only. Additionally, DVT studies were performed using drug-loaded NEBP-NPs at full (3×10^9 ml^{-1}), half (1.5×10^9 ml^{-1}) and double (6×10^9 ml^{-1}) the dose. By comparison, i.v.-free HCQ was used at half the full dose (175 μg per mouse). Treatment with HCQ-loaded NEBP-NPs at half the dose (1/2 × HCQ_NEBP-NPs) led to a significant reduction in thrombus weights compared to no treatment and to free 1/2 × HCQ (Supplementary Fig. 10b). Doubling the dose of HCQ_NEBP-NPs to 6×10^9 ml^{-1} (2 × HCQ_NEBP-NPs) was well tolerated in mice without an increase in mortality and significantly reduced thrombus weights ($P = 0.03$) but added no benefit to thrombus weight reduction when compared to lower doses of HCQ_NEBP-NPs. When comparing PNT-NPs versus PBP-NPs versus NEBP-NPs, we found no significant differences in therapeutic efficacy (Fig. 6h), suggesting that in a milieu where activated neutrophils and platelets are similarly represented, targeting either cell population can be highly efficacious and safer than systemic drug treatment (compare 1/2 × HCQ alone versus 1/2 × HCQ_NEBP-NPs; $P = 0.0065$).

On completion of the DVT studies, we retrieved various clearance organs to assess the NP biodistribution. The RhB signal at 24 hours was significantly low, indicating that a very small percentage dose either cleared or remained in these organ beds (Supplementary Fig. 10c).

Conclusions

Increasing evidence has uncovered significant phenotypic heterogeneity within the neutrophil population. Functional characterization of neutrophils isolated from individuals with autoimmune diseases, chronic infections and cancer identified a distinct subpopulation that exhibits a pro-inflammatory, activated phenotype with prolonged lifespan and susceptibility to form NETs[43]. In this context, we aimed to develop a therapeutic strategy that specifically targets these pathogenic neutrophil subsets without the ubiquitous inhibition of resting cells.

In designing an activation-state-specific NP, we chose to target NE since several pathogenic, pro-inflammatory functions of neutrophils (for example, tissue proteolysis and NET-osis) are in part elastase dependent. Studies with human proteins confirmed that NEBP avidly

binds to hNE but not to PR3, cathepsin G or non-neutrophil proteases. Prior studies have shown that several factors influence serpin behaviour, among them (1) the length of the reactive centre loop, (2) secondary binding sites outside of the reactive centre loop and (3) intramolecular control of the reactive centre loop conformation[44]. The above suggest that the binding kinetics and activity of intact AAT may not be identical to that of reactive-centre-loop-derived peptides such as NEBP.

To our knowledge, this is the first demonstration of specific targeting of activated neutrophils. Other nanomedicine systems have reported neutrophil-specific targeting; however, their design reveals the potential for cross-reactivity with other immune cells. For example, targeting crystallizable fragment (Fc)-gamma receptors[14,15] would also affect B lymphocytes, natural killer cells and macrophages, whereas targeting of scavenger receptors would affect all myeloid cells, as well as dendritic and endothelial cells[45]. Although NE may not be the pathophysiologic driver in all neutrophil-mediated diseases, its cell-specific expression, distinct surface retention on activated neutrophils and presence on NETs has been widely demonstrated. These properties confer NE with a unique ability to serve as 'bait', capturing targeted NPs for localized drug delivery. Considering an average neutrophil lifespan of ~18.5 h (ref. [46]), the NP half-life at steady state would be comparable for meaningful targeting of pathogenic cells. Although in vivo use of liposomal NPs has proven safe[47], further work is required to better determine the pharmacokinetics, elimination and safety profile of NEBP-NPs and PNT-NPs. Trafficking studies showed that intracellular movement of NEBP-NPs was detectable only in the activated group of neutrophils, suggesting that the rate-limiting step for NP internalization is the binding of NEBP to hNE. This would resemble a process of 'receptor-mediated endocytosis', which has been described for hNE interactions with yeast[48].

Finally, we demonstrate therapeutic proof-of-concept studies of drug-loaded PNT-NPs[49]. We provide additional mechanistic insight that HCQ effectively downregulates neutrophil degranulation and NET formation. While HCQ is currently approved in an oral formulation, its systemic use has been linked to several adverse events[39–41]. The present findings should form the basis for future studies to test therapeutic agents that have a limited half-life, a narrow therapeutic window or decreased tissue penetration. Our group has previously shown that the axis of Factor XII and urokinase plasminogen activator receptor upregulates neutrophil functions to lead to enhanced cell migration and NET-osis[50]. Abrogating Factor XII-mediated effects, specifically in neutrophils, improved cutaneous wound healing and restricted ovarian tumour growth and dissemination[50,51]. In non-sterile inflammation, exuberant activation of neutrophils was linked to microvascular thrombosis and tissue injury in severe acute respiratory syndrome coronavirus 2 infection[52]. Therefore, neutrophil-targeted strategies, tailored to treat activated cells and resolve perpetuating inflammation, can potentially be a safe and highly efficient approach to neutralize the harmful pathologic effects driven by neutrophils, promote healing and preserve innate immunity or haemostasis.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information;

details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41565-022-01161-w.

Methods

Animals.

Eight- to 12-week-old male and female mice in a C57BL/6J background (Jackson Laboratories) were equally used for all studies. Animal care and procedures were reviewed and approved by the Institutional Animal Care and Use Committees at Case Western Reserve University (protocol no. 2015-0109) and University of Pittsburgh (protocol no. 21110037) and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Isolation of murine peripheral blood neutrophils.

Murine peripheral blood was drawn by IVC venipuncture into sodium citrate tubes (ratio, one part anticoagulant to nine parts whole blood). Peripheral neutrophils were isolated with a magnetic bead separation system (Miltenyi Biotec) according to the manufacturer's instructions. The eluted cells were resuspended in serum-free medium without growth factors.

Human platelet, plasma and neutrophil isolation.

All human blood sample studies were performed with blood from healthy individuals in accordance with a protocol approved by the Institutional Review Boards (Case 12Z05, IRB no. 09-90-195) of University Hospitals Cleveland Medical Center and the Colorado Multiple Institutional Review Board. All participants provided written informed consent. The protocol, amendments and informed consent forms were approved by the institutional review board. Eligible healthy subjects were 18 years or older; male or female; not on active medications including immunosuppressive and over the counter nonsteroidal anti-inflammatory drugs; and without diagnosis of an acute illness in the past four weeks. Whole blood was drawn by venipuncture from healthy individuals into sodium citrate tubes (ratio, one part anticoagulant to nine parts whole blood). To obtain platelet-rich plasma, whole blood was centrifuged at 150*g* for 15 min at room temperature. Platelet-rich plasma was added to a sepharose, and a HEPES-buffered saline (HBS)-containing column and droplets were collected in fractions to obtain gel filtered platelets, which were counted on a Beckman Coulter Counter. To obtain platelet-poor plasma, whole blood was centrifuged sequentially at 150*g* for 15 min, followed by 2,000*g* for 25 min at room temperature. Platelet-poor plasma supernatants were divided into 0.25 ml aliquots, transferred into clean polypropylene tubes and stored at −80 °C. Peripheral neutrophils were isolated using a magnetic bead separation system (EasySep, Stem Cell Technologies) according to the manufacturer's instructions. Eluted cells were counted using a haemocytometer and trypan blue, centrifuged at 300*g* for 10 min and resuspended in DMEM/F-12 containing 1% bovine serum albumin, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for in vitro functional assays. Platelets were activated with 20 nM thrombin, and neutrophils were activated using 1 μM fMLP, unless otherwise stated.

Ly6G–Fab generation and purification.

Ly6G–Fab generation and purification was done using the Pierce Fab Preparation Kit according to the manufacturer's instructions. Briefly, the process requires equilibration of immobilized papain, preparation of Ly6G IgG samples, generation of antibody fragments and Ly6G–Fab purification steps. To assess digestion completion, we evaluated the digest and wash fractions via non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Following purification, all elutions and flow-through containing Fc and undigested IgG fragments were assessed for Fab content and purity. Protein concentration was measured by absorbance at 280 nm, using an estimated extinction coefficient of 1.4. On average, Fab yields ranged from 45–65% of the starting Ly6G antibody concentration (7.81 mg ml^{-1}).

Studies with NPs.

Confocal microscopy.—NP binding was assessed in vitro by confocal imaging using 5×10^5 human neutrophils and 1×10^7 platelets. First, platelets were stained with calcein AM (1 μg ml^{-1} final concentration) prior to incubation with neutrophils or NPs. Next, neutrophils, platelets and 3×10^9 ml^{-1} NPs (U-NP, NEBP-NP, PBP-NP or PNT-NP) were mixed before being added to slides and were incubated for 2 hours at 37 °C in the presence of 1 μM fMLP and 20 nM thrombin. On completion of incubation, slides were gently washed with PBS, fixed with 4% paraformaldehyde for 5 min, washed again with PBS, mounted with VectaShield DAPI mounting solution and cover-slipped. Slides were imaged using a Leica HyVolution SP8 confocal microscope, and fluorescent intensity was quantified using ImageJ 2.0.0 software (National Institutes of Health).

Flow cytometry.—Flow cytometry experiments used 1×10^6 ml^{-1} neutrophils and 3×10^9 ml^{-1} NPs per tested condition. Human neutrophils were either left untreated or were stimulated with 1 μM fMLP and incubated with U-NPs or PNT-NPs for 1 hour at 37 °C. Following a washing step with PBS, cells were fixed with 1% paraformaldehyde for 15 min at room temperature. After additional washing steps, samples were centrifuged at 600*g* for 10 min, resuspended in 400 μl of PBS and placed on ice until analysis. Neutrophils were gated based on forward and side scatter characteristics. At least 10,000 cells gated from forward and side scatter characteristics were acquired for analysis, and RhB fluorescent intensity (detection channel YG610A) was measured for each group using a LSR II flow cytometer (BD Biosciences). Images were acquired for analysis using BD FACSDiva software (BD Biosciences). Data analysis was performed with FlowJo 10.4.2 software. Similarly, human platelets (1×10^7 ml^{-1}) were either left untreated or were activated with 20 nM thrombin and incubated with PNT-NPs. Cells were first sorted by scatter and CD42b positivity (B525A; 1:100 dilution). Then, activated platelets were gated by CD62P+ (1:100 dilution; detection channel R600A), followed by gating for RhB fluorescence. For studies with NEBP-NPs, purified human neutrophils were treated with or without 10 ng ml^{-1} TNF-α, 5 μg ml^{-1} LPS or 1 μM fMLP and coincubated with RhB-labelled NEBP-NPs for 1 hour at 37 °C. Following a washing step with PBS, cells were fixed with 1% paraformaldehyde for 8 min at room temperature. After additional washing steps, samples were centrifuged at 600*g* for 10 min, resuspended in 400 μl of FACS buffer and placed on ice until analysis. Cells were gated based on forward and side scatter characteristics and initially sorted by

CD66b (AF700; 1:100 dilution) positivity, followed by doublet discrimination and gating for NE (Cy5; 1:100 dilution). The percentage of CD66b and NE double positive single cells in complex with NEBP-NPs was determined by measuring RhB fluorescent intensity (YG610A).

Additional methods are provided in the Supplementary Information.

Statistical analysis.

All data are presented as mean ± standard error of the mean (s.e.m.) unless otherwise indicated and with the indicated sample size. Difference between two groups was determined by unpaired two-tailed Student's *t*-test. One-way ANOVA analysis with Bonferroni post-hoc analysis was used to compare three or more related groups. To determine if soluble P-selectin interferes with PBP-NP binding to surface-exposed P-selectin, we employed ordinary one-way ANOVA with Dunnett's multiple comparisons test. NP half-life and PNT-NP binding to neutrophils and platelets (flow cytometry) were calculated with ordinary two-way ANOVA. Prism 9.0.0 (GraphPad), FlowJo 10.4.2, ImageJ 2.0.0, CellSens v.1.18, MO.Affinity Analysis v.2.3 and SoftMax Pro VS.4.5 software were used for analysis. A P value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Modelling of docking complex with highlighted reactive centre loop and NEBP was performed using native AAT (Protein Data Bank code 1QLP)[20,21]. The authors declare that data supporting the findings of this study are available in their entirety within the article and its Supplementary Information. Relevant data can be provided by the corresponding authors upon reasonable request.

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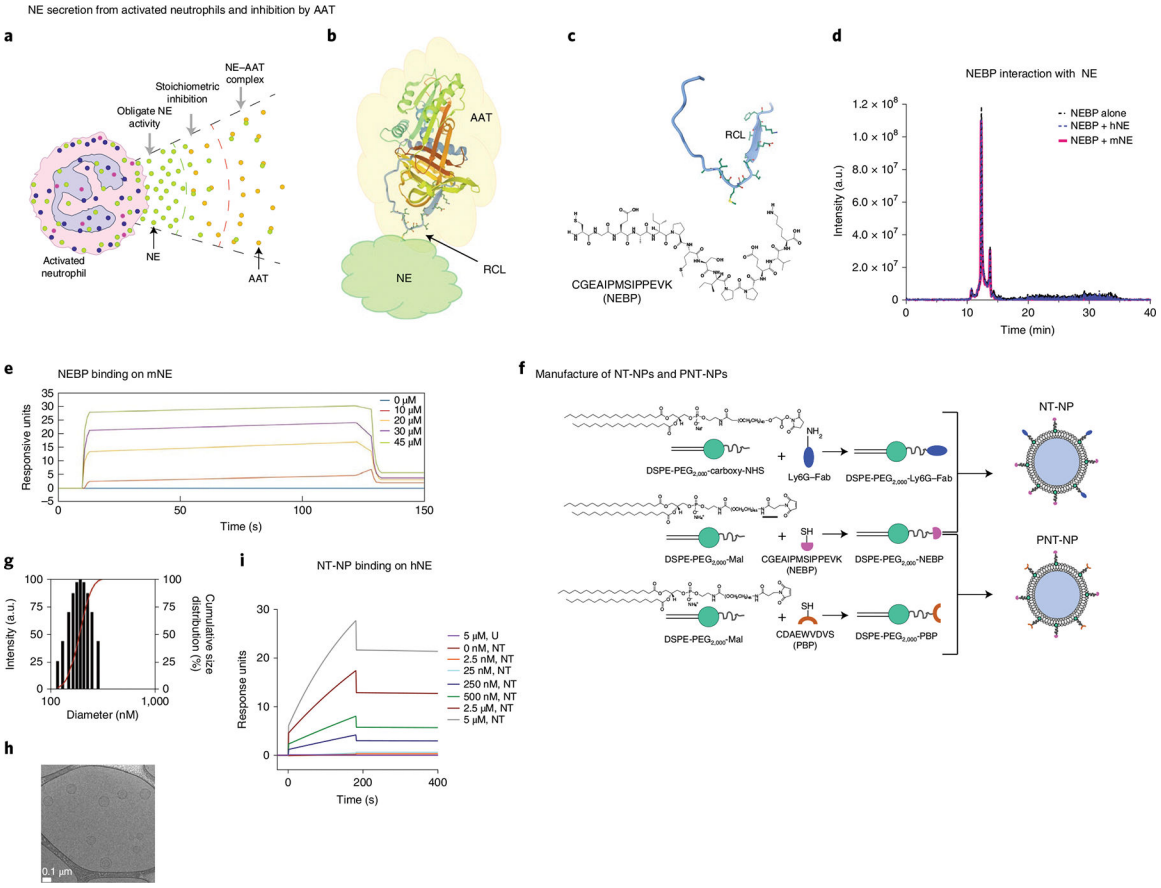

Fig. 1 |. Design and characterization of NPs for selective targeting of activated neutrophils.

a, Conceptual framework of targeting NE as a strategy to differentiate activated from resting neutrophils. **b**, Physiologically, NE recognizes and binds to a 20 amino acid sequence within the reactive centre loop (RCL; magnified in **c**) of AAT. **c**, A NEBP was designed from the reactive centre loop of AAT. A cysteine at the P7 position was added for subsequent peptide conjugation to NP lipids. Modelling of docking complex with highlighted reactive centre loop and NEBP was performed using native AAT (Protein Data Bank code 1QLP)[20,21]. **d**, Solution-phase hydrolysis of NEBP by NE. High-performance liquid chromatography chromatogram showing pure NEBP (black dashed line) and combination of NEBP with mouse (mNE, red line) or human (hNE, blue dashed line) NE reaction mixture. Data are representative of $n = 3$ individual experiments run in triplicate. **e**, Surface plasmon resonance of NEBP binding to immobilized mNE. Increasing concentrations of NEBP were injected over a mNE-immobilized CM5 chip. **f**, To exclusively target activated neutrophils, Ly6G–Fab (blue) was conjugated to DSPE-PEG-carboxy-NHS; NEBP (purple) was conjugated to DSPE-PEG-Mal. To simultaneously target activated platelets and neutrophils, NEBP and PBP were conjugated to DSPE-PEG-Mal to derive PNT-NPs. DSPE-PEG-NHS, N-hydroxysuccinimide terminated polyethylene glycol conjugated distearyl phosphoethanolamine; Mal, maleimide. **g**, NP size was determined by dynamic light scattering; mean = 200 nm. **h**, Molecular resolution of NPs using cryogenic transmission electron microscopy; $n = 3$ individual experiments. Scale, 0.1 μm. **i**, Kinetic

real-time binding of NT-NPs (NT; 0–5 μM) or U-NPs (U; 5 μM) to immobilized hNE; $n = 3$ individual Langmuir binding models run in triplicate.

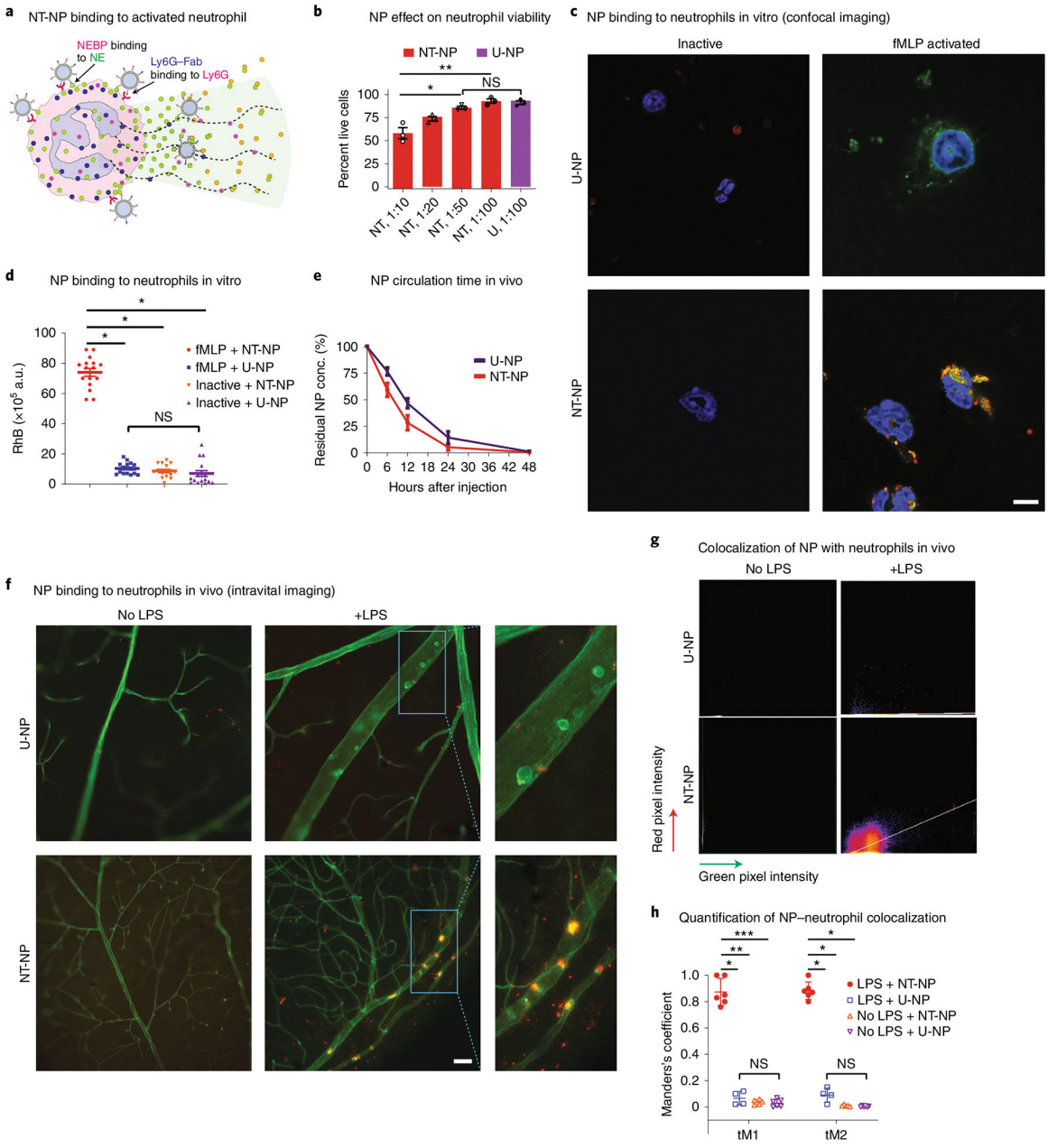

Fig. 2 |. NT-NPs selectively bind to activated neutrophils in vitro and in vivo.

a, Schematic representation of targeting ligands on NT-NPs and their binding targets on activated neutrophils. **b**, Neutrophil viability in the presence of rising concentrations of NT-NPs or U-NPs. Mean ± s.e.m., $n = 3$ individual experiments run in triplicate, $*P = 0.0009$, $**P = 0.0001$, one-way analysis of variance (ANOVA) with Bonferroni correction. NS, not significant. **c**, Confocal microscopy of static murine neutrophils incubated with NT-NPs or U-NPs, in the absence or presence of fMLP. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) and NE (green). NPs were labelled with RhB (red). Scale, 10 μm; $n = 3$ independent experiments. **d**, NP binding on neutrophils shown in **c** was quantitated by measuring the residual RhB fluorescence on cells following several washing steps; n

= 3 independent experiments. Symbols denote high-power fields captured per condition (n = 16). Mean ± s.e.m., *P < 0.0001, one-way ANOVA with Bonferroni correction. **e**, Wild-type mice (n = 3 per time point) were i.v. administered U-NPs or NT-NPs, and residual NP concentration (conc.) was determined by a RhB fluorescence assay. Mean ± s.e.m., n = 3 independent experiments. **f**, Mice were i.v. injected with saline or 1 mg kg^{-1} body weight *Escherichia coli* O111:B4 LPS. Three hours after, anti-Gr-1 antibody (green) mixed with RhB-labelled NPs (U-NPs or NT-NPs) were i.v. injected and allowed to circulate for 40 min before retinas were excised. Retinal vasculature was visualized with Concanavalin A conjugated to fluorescein isothiocyanate. Square and dotted lines in the middle panels demarcate the magnified retinal regions in the right panels. LPS + NT-NP, n = 6 mice; LPS + U-NP, n = 4 mice; no LPS + NT-NP, n = 5 mice; no LPS + U-NP, n = 6 mice. Scale, 50 μm. **g**, Representative two-dimensional intensity histograms from retinal images. The y axis represents above-zero red pixel intensity (RhB-labelled NPs). The x axis indicates above-zero green pixel intensity (adhered neutrophils). **h**, Colocalization analysis of retinal images shown in **f**. Thresholded Manders's correlation coefficient values (tM1 = red overlap with green; tM2 = green overlap with red) are shown among groups. Costes probability value ≥ 95% in LPS + NT-NP; Costes probability value = 0% in all other groups. LPS + NT-NP, n = 6 mice; LPS + U-NP, n = 4 mice; no LPS + NT-NP, n = 5 mice; no LPS + U-NP, n = 6 mice. Mean ± s.e.m., *P < 0.000001, **P = 0.000002, ***P = 0.000003, one-way ANOVA with Bonferroni correction.

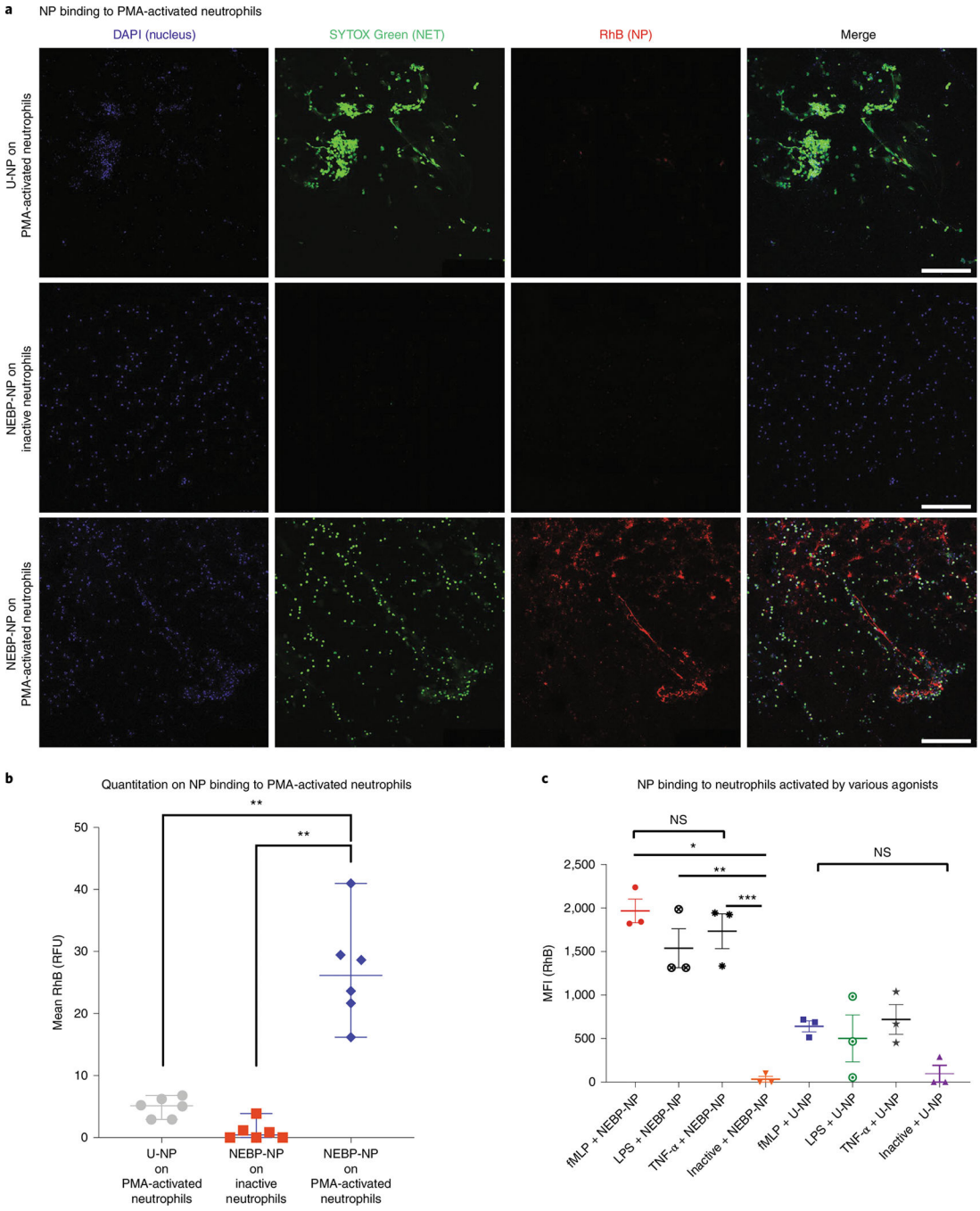

Fig. 3 |. NT-NPs selectively bind to activated neutrophils and NETs.

a, Freshly isolated human neutrophils were treated with media (inactive) or activated with 100 nM PMA in the absence or presence of 3×10^9 ml^{-1} U-NPs or (neutrophil-targeted) NEBP-NPs for 2 h. Cells were subsequently stained with DAPI (blue, nuclei) and SYTOX Green (1 μM, green, extracellular DNA); fixed with 4% formalin for 4 min; and washed in PBS but not permeabilized. NPs are inherently red due to RhB labelling. Fluorescent images were obtained using a Leica TCS SP8 confocal microscope at ×10 magnification. Images are representative of $n = 6$ individual experiments. Scale, 250 μm. **b**, Quantitation of NP binding

to inactive or PMA-activated neutrophils. Mean ± s.e.m., $n = 6$ individual experiments, $*P = 0.001$, $**P = 0.0022$, one-way ANOVA with Bonferroni correction. RFU, relative fluorescence units. **c**, Purified human neutrophils were treated with media or neutrophil agonists TNF-α (10 ng ml^{-1}), LPS (5 μg ml^{-1}) and fMLP (1 μM). Cells were coincubated with 3×10^9 ml^{-1} RhB-labelled U-NPs or NEBP-NPs for 1 hour at 37 °C. After several washing and centrifugation steps, cells were fixed and stained with CD66b Alexa Fluor 700 (AF700) and NE Cy5 antibodies. To measure neutrophil–NP binding, cells were first sorted by CD66b positivity, followed by doublet discrimination and gating for hNE. The RhB fluorescence of CD66b and NE double positive single cells was measured. Mean ± s.e.m., $n = 3$ individual experiments, $*P = 0.0015$, $**P = 0.01$, $***P = 0.0046$, one-way ANOVA with Bonferroni correction. MFI, mean fluorescence intensity.

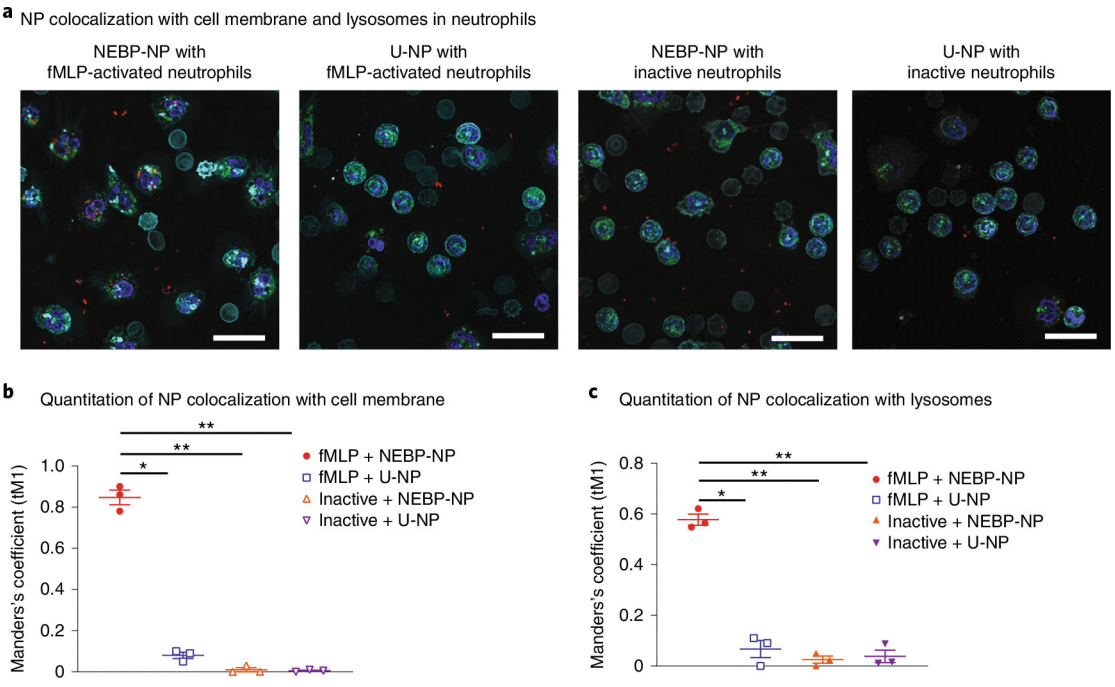

Fig. 4 |. Quantitation of NP trafficking by neutrophils.

a, Human neutrophils (1×10^6 ml^{-1}) were resuspended in serum-free Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12) containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, plated in 35 mm glass-bottom dishes and allowed to adhere for 15 minutes. Where indicated, cells were activated with 1 μM fMLP in the absence or presence of 3×10^9 ml^{-1} U-NPs or (neutrophil-targeted) NEBP-NPs at 37 °C for 2 h. Cells were maintained alive and were not fixed or permeabilized. On completion of the incubation time, cell components were stained and imaged. Scale, 20 μm; $n = 3$ individual experiments. **b**,**c**, For colocalization of NPs with cellular components, thresholded Manders's correlation coefficient values (tM1) are shown among groups for cell membrane–NP interactions (**b**) and lysosome–NP interations (**c**). Costes probability value ≥ 95% in fMLP + NEBP-NP; Costes probability value = 0% in all other groups; $n = 3$ individual plates scanned; area, 200 μm × 200 μm. For **b**, mean ± s.e.m., $*P = 0.001$, $**P < 0.001$, one-way ANOVA with Bonferroni correction. For **c**, mean ± s.e.m., $*P = 0.018$, $**P = 0.00092$, one-way ANOVA with Bonferroni correction.

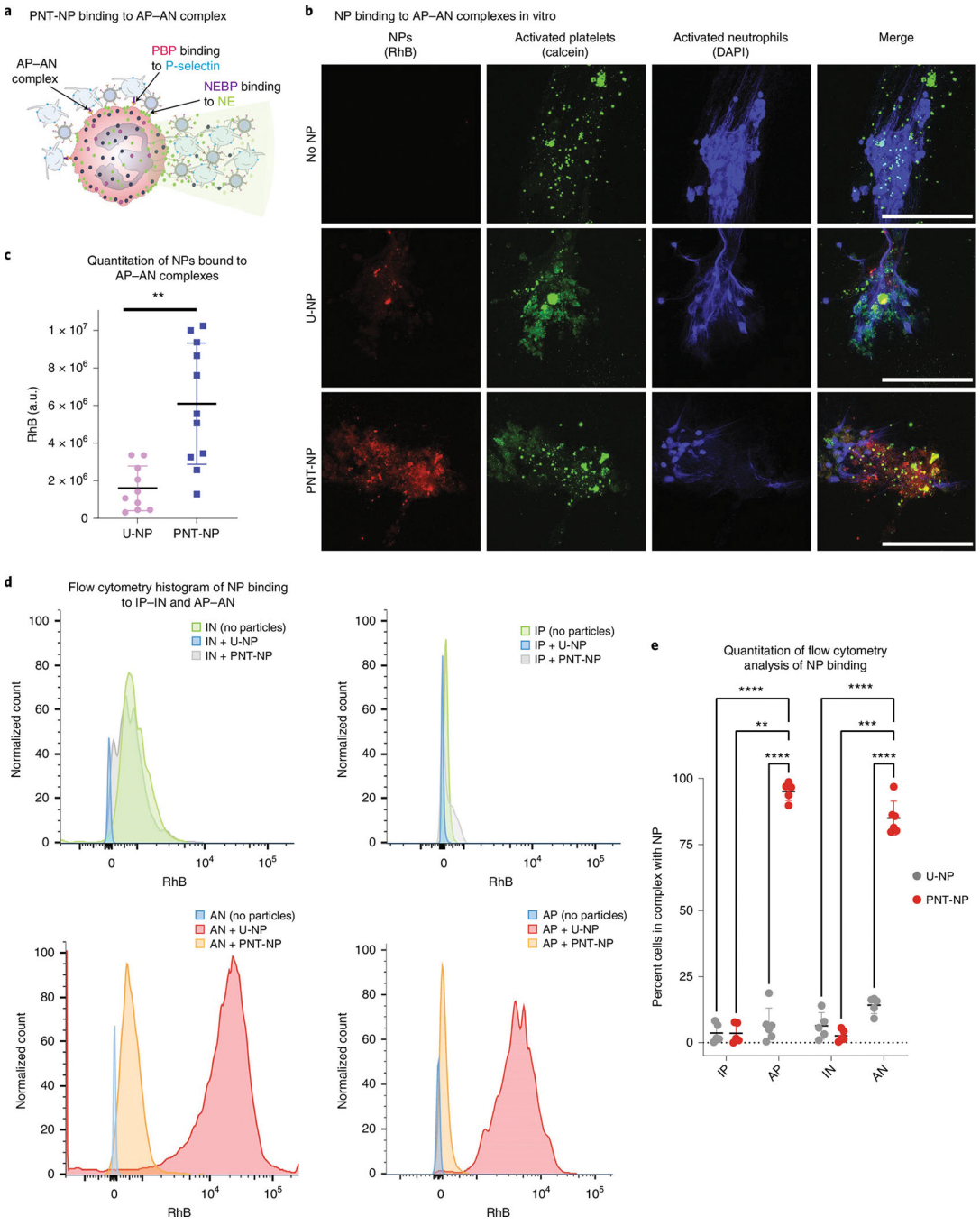

Fig. 5 |. Heteromultivalent NPs selectively bind to activated platelet–neutrophil complexes in vitro.

a, Schematic illustration of PNT-NPs. The combined decoration of NPs with a PBP (targeting P-selectin) and NEBP (targeting NE) enables PNT-NPs to bind simultaneously and selectively to activated platelet–neutrophil complexes. **b**, Freshly isolated human neutrophils and platelets were activated with 1 μM fMLP and 20 nM thrombin prior to being coincubated with RhB-labelled (red) U-NPs or PNT-NPs. Platelets were stained with calcein (green), and nuclei corresponding to activated neutrophils were counterstained with DAPI

(blue). Representative images show that PNT-NPs (third row panels) bound to activated platelet–neutrophil aggregates, but undecorated U-NPs (second row panels) did not. Images are representative of $n = 10–11$ individual experiments run in triplicate. Scale, 100 μm. **c**, NP retention on activated platelet–neutrophil complexes was measured by quantitating residual RhB fluorescence. U-NP, $n = 10$ individual samples; PNT-NP, $n = 11$ individual samples; all run in triplicate. Mean ± s.e.m., $^{**}P < 0.01$ by Student's *t*-test. **d**, Human neutrophils were either left untreated or activated with fMLP and incubated with U-NPs or PNT-NPs. Representative flow cytograms (left upper and left lower panels) of NP retention on inactive (IN) or activated (AN) neutrophils; $n = 5–6$ individual experiments run in triplicate. Human platelets were left untreated or activated with 20 nM thrombin, in the absence or presence of U-NPs or PNT-NPs. Representative flow cytograms (right upper and right lower panels) of $n = 5–6$ individual experiments run in triplicate. AP, activated platelets. **e**, Cell–NP binding to IN, AN, IP or AP was quantitated by measuring RhB fluorescence intensity; $n = 5–6$ individual experiments run in triplicate. Data are presented as mean ± s.e.m., $^{**}P < 0.0017$, $^{***}P = 0.0008$, $^{****}P < 0.0001$, ordinary two-way ANOVA.

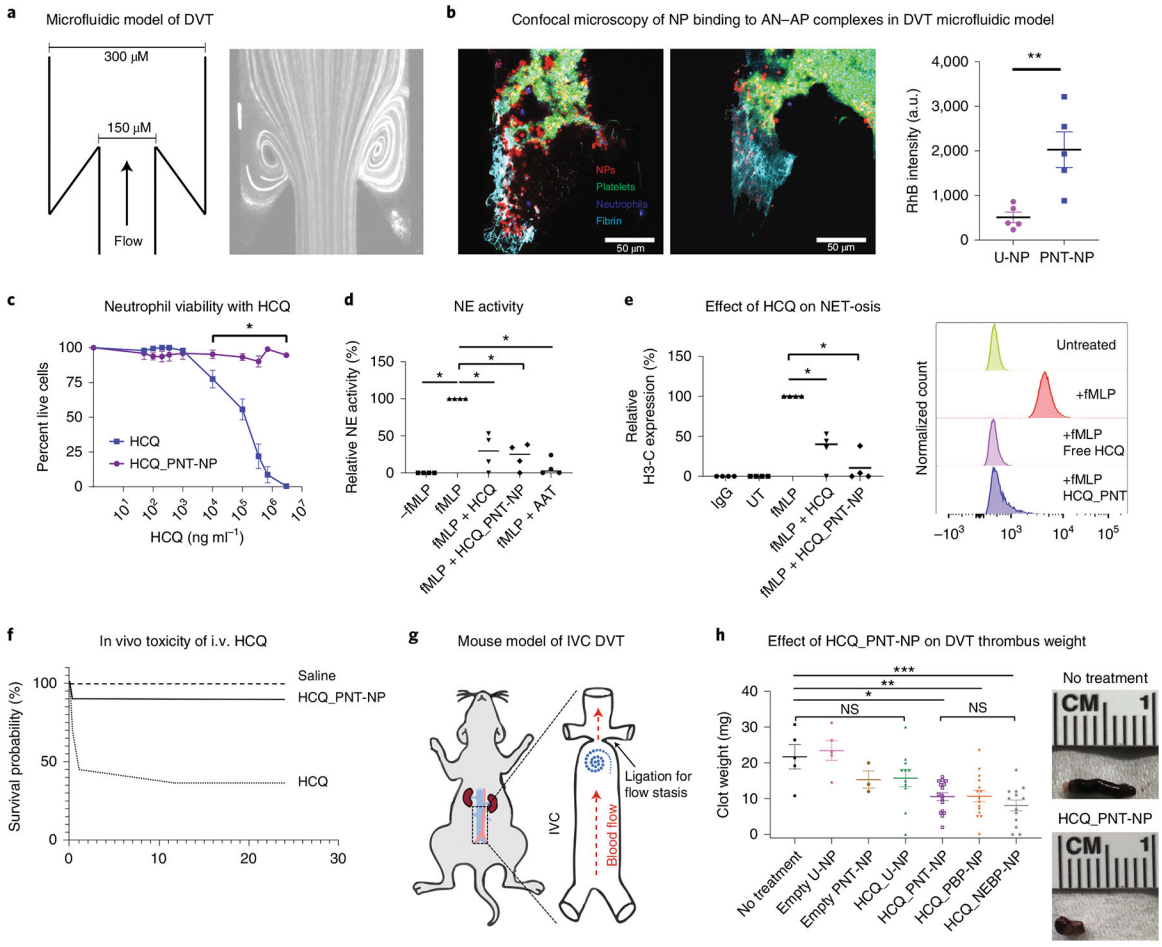

Fig. 6 |. PNT-NPs bind to thromboinflammatory sites and effectively deliver therapeutic cargo to reduce thrombus size.

a, Schematic of microfluidic set-up. Image of 1 μm polystyrene particle streak lines demonstrating vorticle flows recirculating in valve pockets. **b**, Maximum projections of confocal stack after 30 min of blood flow. NPs are in red, human platelets in green, neutrophils in blue and fibrin(ogen) in cyan. RhB fluorescent intensity within microchannels is shown at the right. Mean ± s.e.m., $n = 3$ individual experiments run in triplicate, $^{*}P = 0.006$, two-sided Student's t-test. **c**, Neutrophil viability in the presence of free HCQ or HCQ_PNT-NPs. Mean ± s.e.m., $n = 3$ individual experiments run in triplicate, $^{*}P = 0.0079$ compared to initial (100%) viability, ordinary two-way ANOVA. **d**, NE activity of human neutrophils pretreated with AAT, free HCQ or HCQ_PNT-NPs for 2 h prior to stimulation with fMLP. Mean ± s.e.m., $n = 3$ individual experiments run in triplicate, $^{**}P < 0.0001$, one-way ANOVA with Bonferroni correction. **e**, Flow cytometry analysis of NET formation in human neutrophils pretreated with free HCQ or HCQ_PNT-NP prior to stimulation with fMLP. UT denotes untreated neutrophils. Data are presented as relative expression of citrullinated histone H3 (H3-C). Mean ± s.e.m., $n = 4$ experiments, $^{*}P < 0.0001$, one-way ANOVA with Bonferroni correction. **f**, Kaplan–Meier survival analysis in mice i.v treated with saline (control), free HCQ or HCQ_PNT-NPs. **g**, Schematic representation of deep venous thrombosis model. **h**, Prior to IVC ligation, mice were i.v. injected with saline (no

treatment, $n = 5$); empty U-NPs ($n = 5$); empty PNT-NPs ($n = 3$); HCQ_U-NPs ($n = 10$); HCQ_PNT-NPs ($n = 16$); HCQ_NEBP-NPs ($n = 13$); or HCQ_PBP-NPs ($n = 13$). Thrombi were harvested at 24 h. Each symbol represents an individual mouse. Mean ± s.e.m., $*P = 0.0065$, $**P = 0.01$, $***P = 0.004$, one-way ANOVA with Bonferroni correction. Gross images of harvested thrombi from control (top panel) and mice i.v. treated with HCQ_PNT-NPs (bottom panel).