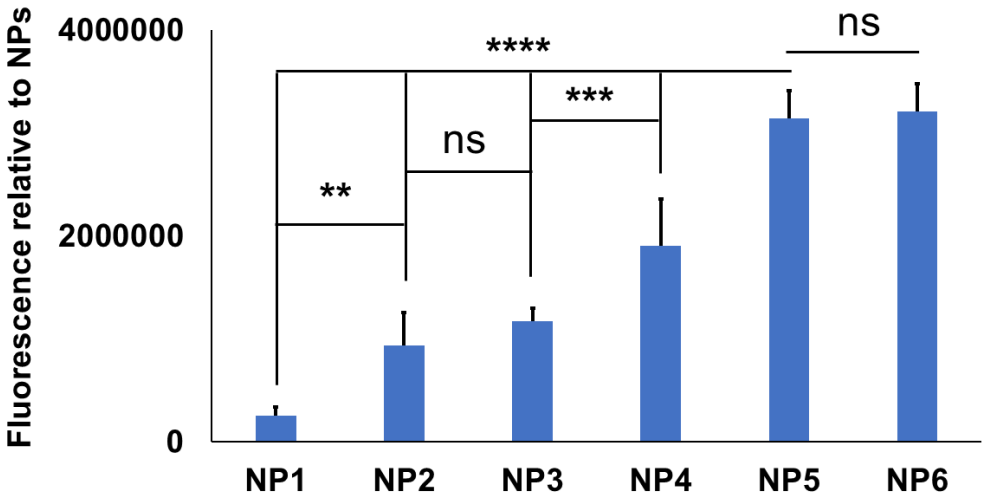
Targeted delivery of Protein Arginine Deiminase-4 inhibitors to limit arterial intimal NETosis and preserve endothelial integrity

Roberto Molinaro[a, b, 1], Mikyung Yu[c, 1], Grasiele Sausen[a], Colette A. Bichsel[d], Claudia Corbo[c,e], Eduardo J. Folco[a], Gha Young Lee[c], Yuan Liu[c], Yevgenia Tesmenitsky[a], Eugenia Shvartz[a], Galina K. Sukhova[a], Frederik Kloss[a], Kevin J. Croce[a], Omid C. Farokhzad[c], Jinjun Shi[c, 2], Peter Libby[a, 2]

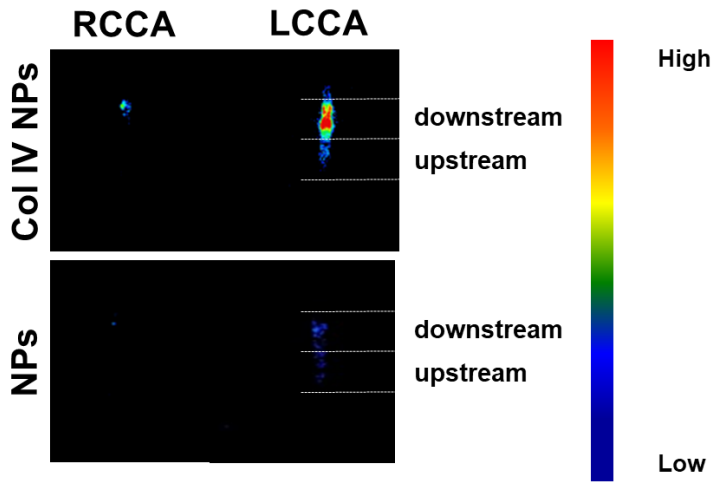
SUPPLEMENTAL MATERIAL

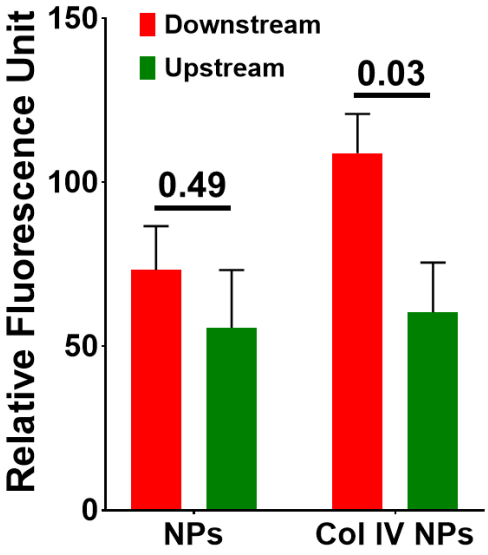
Supplementary Figure 1

Legend	Heptapeptide density (%)
NP1	-
NP2	1
NP3	5
NP4	7.5
NP5	15
NP6	30

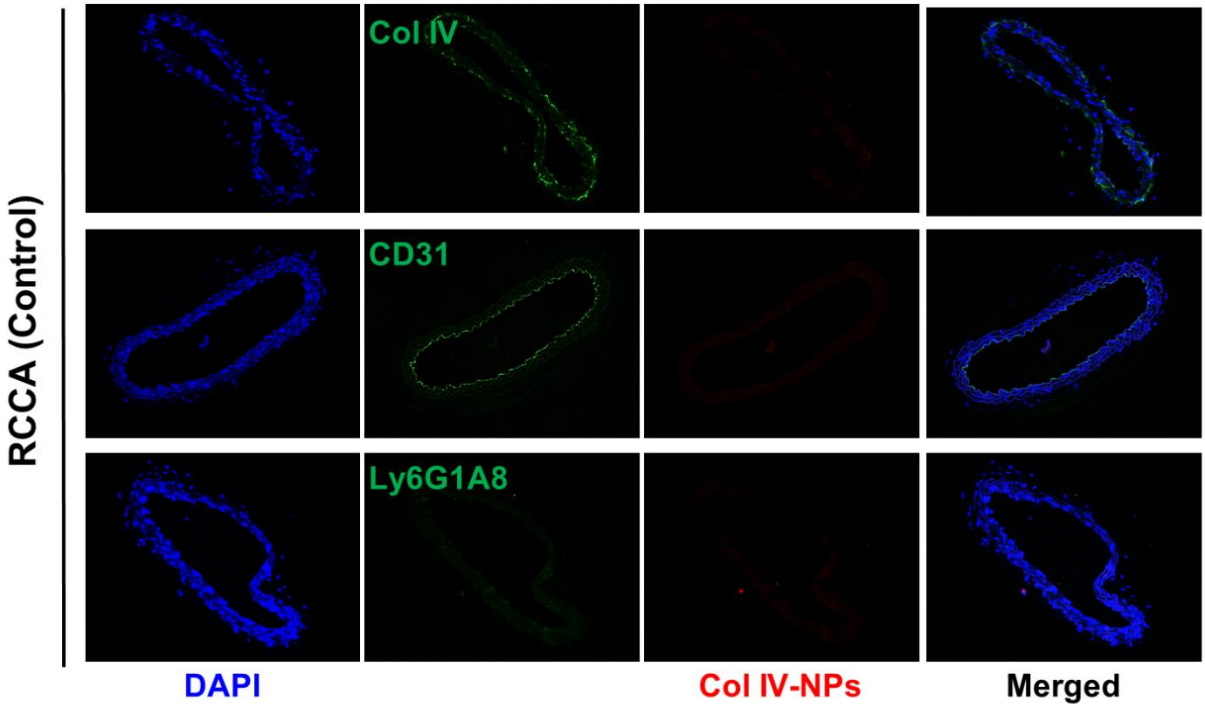


Supplementary Figure 2

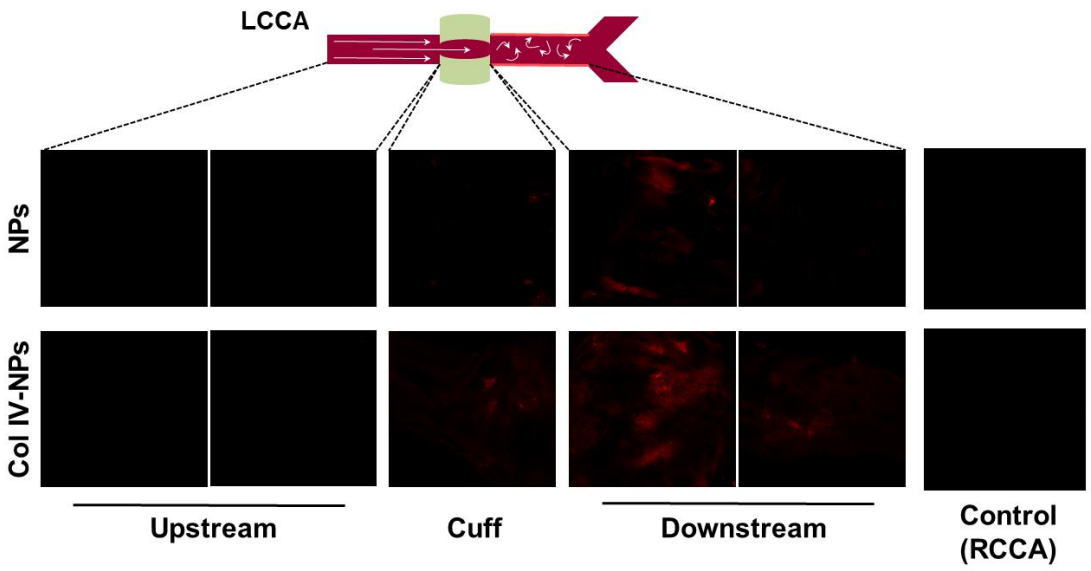




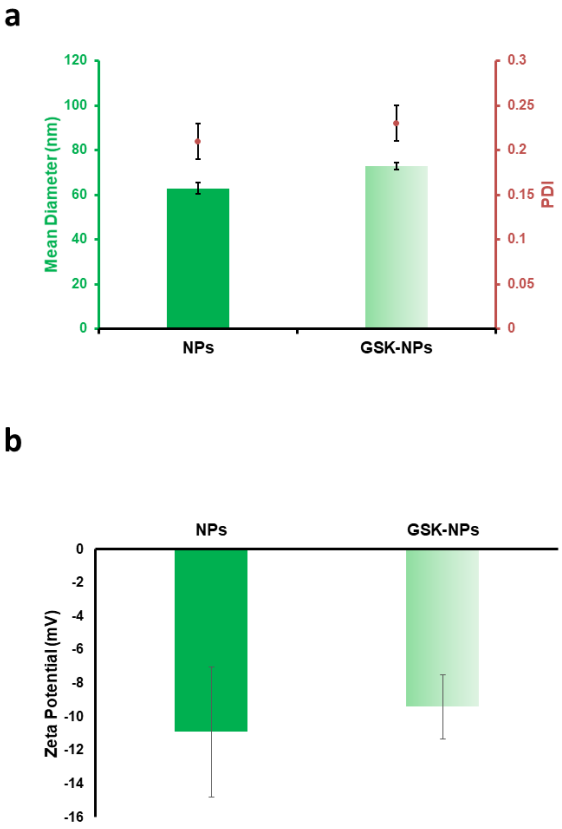
Supplementary Figure 3



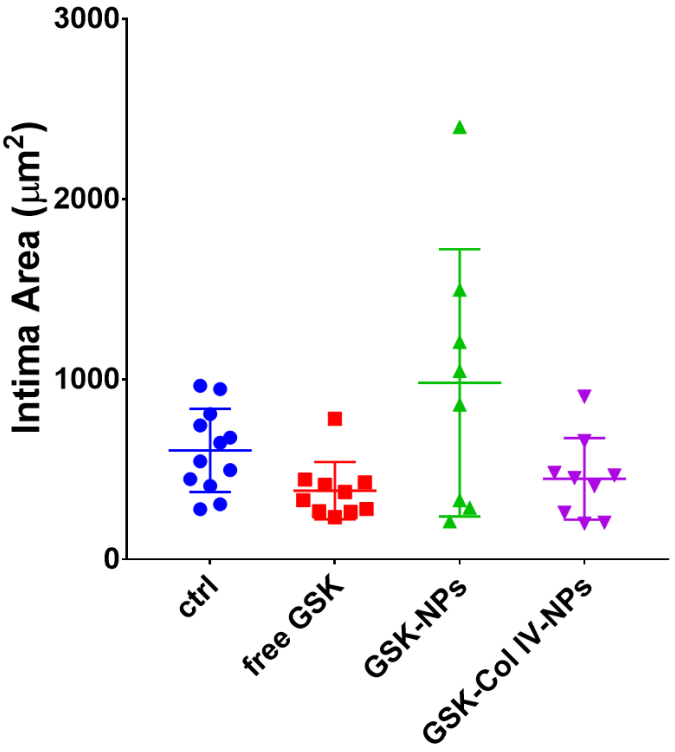
Supplementary Figure 4



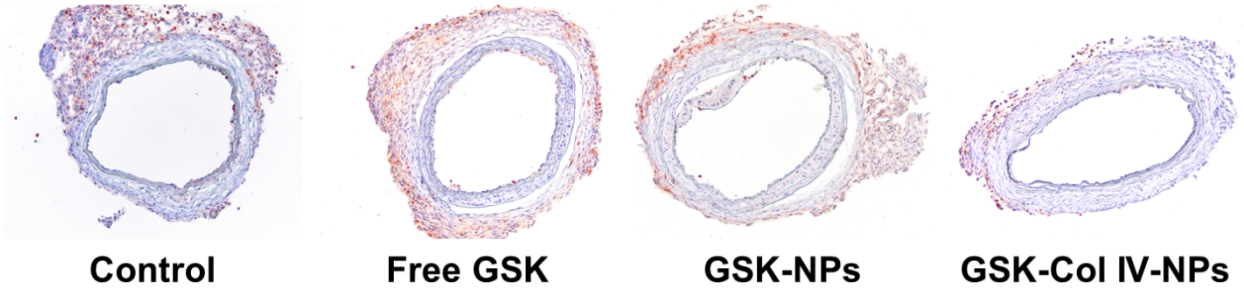
Supplementary Figure 5

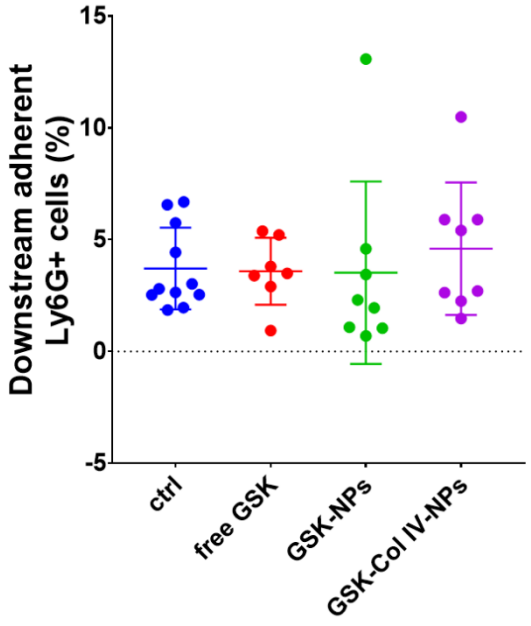


Supplementary Figure 6

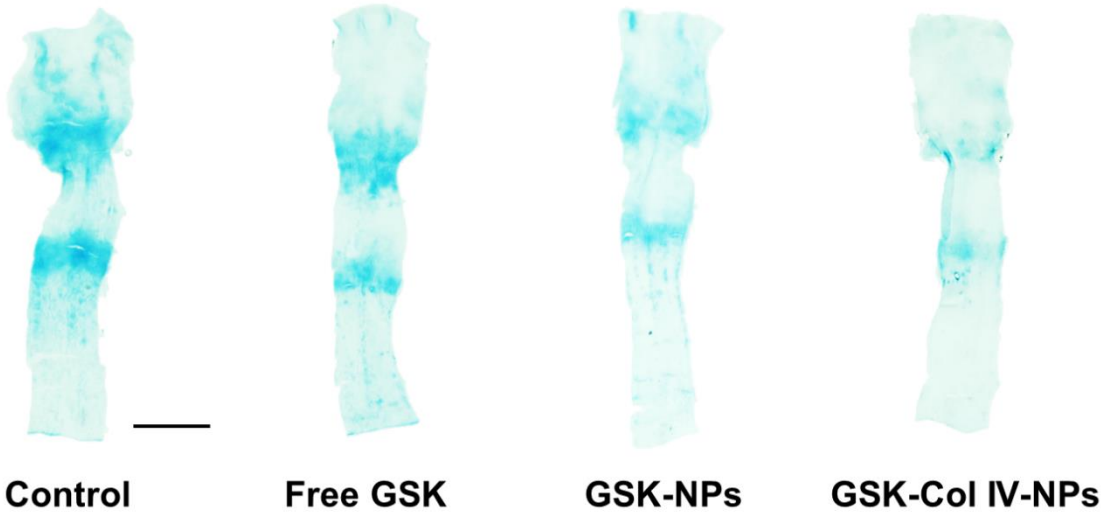


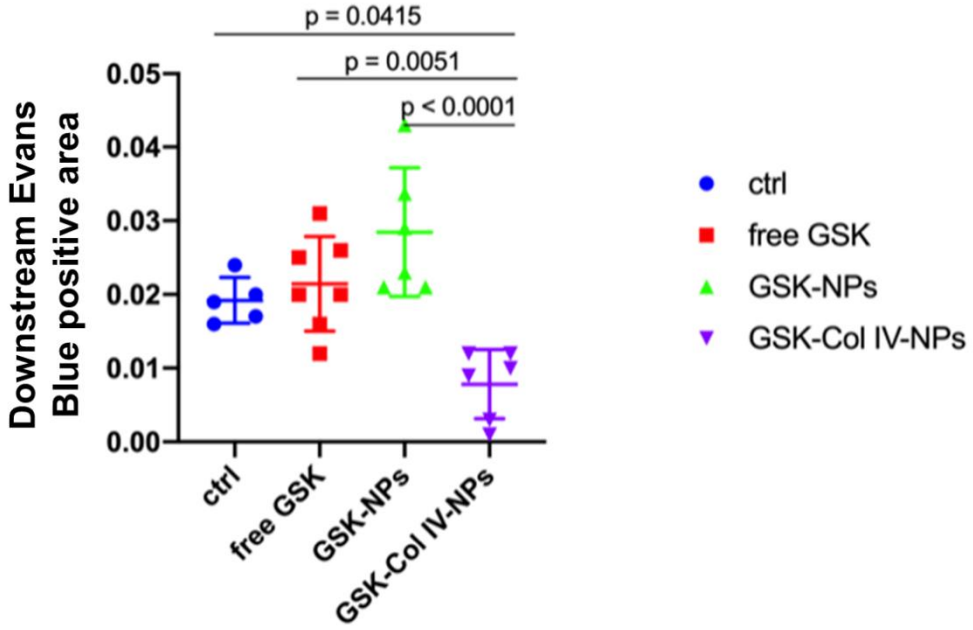
Supplementary Figure 7





Supplementary Figure 8





Supplementary Figure 1 In Vitro Adhesion of NPs to a Collagen IV-coated surface.
The increase of Col IV heptapeptide density on NP surface induces an increase of Col IV NPs adhesion to a Col IV-coated surface. However, no difference was observed between a density of 15 and 30%, thus supporting the use of 15% for further experiments.

Supplementary Figure 2 Fluorescence reflectance images of RCCA (healthy carotid) and downstream and upstream of LCCA (eroded plaque) in mice injected with bare and Col IV-NPs at 24h post-injection. Quantitative assessment of fluorescence intensities of bare and Col IV-NPs reveals higher accumulation of Col IV-NPs downstream of LCCA compared to upstream ($p=0.03$). No significant different between downstream and upstream of LCCA was observed for bare NPs ($p=0.49$).

Supplementary Figure 3 Immunofluorescent staining of Col IV, CD31, and neutrophils (in RED) shows no Col IV-NPs (in RED) localization in RCCA (uninjured carotid).

Supplementary Figure 4 Representative en face distribution of fluorescently labeled NPs and Col IV-NPs (*red*) to the previously injured LCCA subjected to flow perturbation. Upstream, cuff, and downstream regions of LCCA are illustrated.

Supplementary Figure 5 Physicochemical characterization of non-targeted NPs. **a**) Dynamic light scattering (DLS) analysis of empty and GSK484 (GSK)-loaded NPs reveals a slight increase of the hydrodynamic size after GSK encapsulation (63 and 73 nm for empty and GSK-NPs, respectively). GSK encapsulation did not significantly affect the homogeneity of the formulations, as indicated by the polydispersity index values before and after GSK encapsulation (PDI=0.21 and 0.23, respectively). **b**) Zeta potential analysis reveals a negative surface charge for both empty and GSK-loaded NPs, without significant changes after GSK encapsulation.

Supplementary Figure 6 Measurement of intima areas of previously injured LCCA subjected to flow perturbation. The groups (n=8-12) did not differ significantly, thus indicating the reproducibility of the experimental procedure.

Supplementary Figure 7 PAD-4 inhibition through either free or encapsulated GSK484 does not affect neutrophil recruitment downstream the flow perturbation. Immunohistochemical evaluation of previously injured LCCA subjected to flow perturbation from mice (n=8-12) treated with GSK484 (GSK), either alone or encapsulated in bare (GSK-NPs) and Col IV NPs (GSK-Col IV-NPs), stained with anti-Ly6G antibody (neutrophils marker), showed no significant difference in the percentage of adherent neutrophils at sites of eroded plaques.

Supplementary Figure 8 Representative images (upper panel) of Evans blue exclusion analysis of previously injured (electric injury plus flow disturbance induced by constrictive cuff placement) carotids from ApoE$^{-/-}$ mice (n=5-7 per group), untreated (Control) or treated with free, unencapsulated GSK484 (Free GSK) or encapsulated in either bare nanoparticles (GSK-NPs) or Collagen IV-targeting NPs (GSK-Col IV-NPs). Evans Blue coverage analysis reveals impaired barrier function for all groups after flow perturbation. GSK-Col IV-NPs treated mice showed a reduced permeability of the endothelial barrier compared to the other groups (lower panel), thus indicating a a beneficial activity of GSK-Col IV-NPs in preserving endothelial continuity and ameliorating the endothelial damage. Scale bar = 500 μm.