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Supplementary Materials for

Neutrophil-Derived Cathelicidin Protects from Neointimal Hyperplasia

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The PDF file includes:

Materials and Methods

Immunohistochemistry

Left common carotid arteries were excised after perfusion–fixation with paraformaldehyde (4%) in situ. Serial tissue sections (5 µm) were obtained starting at the bifurcation. Movats-modified pentachrome staining was used to determine neointimal areas. Sections were reacted with antibodies to Mac-2 (Cedarlane), α-SMC actin (Dako), CD3 (Serotec), Ly6G, CD31 (Abcam) or KI-67 (Imgenex). Gomori trichrome stain was used to assess collagen content.

Intravital microscopy

Neutrophil adhesion to injured carotid arteries was analyzed by intravital microscopy in *Lysm*$^{egfp/egfp}$*Apoe*$^{-/-}$ mice depleted of monocytes by chlodronate liposomes (23,27). For luminal detection of CRAMP presented on endothelium, protein G Fluoresbrite® YG microspheres (Polysciences) were coupled to anti-CRAMP polyclonal antibody (Innovagen) (23). To assess adhesion, 10^6 DiI-labeled EOCs were injected i.v. 4 h after injury. Recordings were made 15 min after injection. Intravital and 2-photon microscopy was performed as described (23).

Cell proliferation and apoptosis

For cell-cycle analysis, cells were fixed in 70% ethanol and stained with propidium iodide (30 µg/ml) in 0.25% triton-X100 in PBS containing RNase A (4 µg/ml). To induce apoptosis, TNF was added to EOC culture medium with or without LL-37. After 12 h, cells were detached and incubated with FITC-conjugated annexin-V.

Flow cytometry

EOCs were reacted with antibodies against CXCR4, CD14, Tie-2, CD11b (BD Pharmingen), CD31 (eBioscience), VE-cadherin, CXCR2, FPR2 (R&D Systems), or isotype-matched IgG1 or IgG2a controls. Flow cytometry was performed using a FACSCanto II (BD Biosciences).

Real-time PCR analysis

DNA-free total RNA was extracted from EOCs using RNeasy Mini-kit (Qiagen). Reverse transcription was performed at 42°C for 15 min and terminated at 95°C for 1 min. cDNA fragments were amplified by 45 cycles of PCR (denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 2 min); Following primers were used: FPR2-forward 5'-TTGGTTTCCCTTTCAACTGG-3', FPR2-reverse 5'-ACGTAAAGCATGGGGTTGAG-3', GAPDH-forward 5'-GACCTTCATTGACTCAACTACA-3', GAPDH-reverse 5'-GCCCCTCCCCTCTTCAAG-3'.

Flow assay

Dishes were coated with laminin (10 µg/mL), fibrinogen (10 µg/mL) or vitronectin (1 µg/mL; Sigma-Aldrich). Confluent HAoECs were activated with TNF (50 ng/ml) and LL-37 (1 µg/ml) was added 15 min before assembly in a parallel-wall flow chamber. EOCs labeled with calcein were perfused at 1.5 dyne/cm^2 in the presence or absence of boc-PLPLP (1 µM).

Cell culture

To prepare CM, EOCs were washed and cultured in RPMI-1640/1% FCS with or without LL-37 (1 µg/ml). After 24 h, medium was collected, centrifuged and passed through a filter. Residual LL-37 was immunodepleted using anti-LL-37 antibodies coupled to protein G beads.

Binding of LL-37 to HAoECs was assessed by FACS analysis. LL-37 (1 µg/ml) was incubated with HAoECs for 15 min and stained with rabbit anti-LL-37 polyclonal Ab (Santa Cruz) in 1% BSA. HAoECs were pre-treated with heparinase II (50 U/ml), chondroitinase$_{ABC}$ (20 U/ml, Sigma-Aldrich), or cyclohexamide (500 ng/ml, Sigma).

Circularity measurements were performed using Image J software. Analyses is based on the following equation: $circularity = 4\pi(area/perimete^{\wedge 2})$. A circularity value of 1.0

indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

Stent braiding, coating, implantation, and processing

Stents were manually braided from sixteen 50µm Nitinol wires yielding an outer diameter of 500µm and heated in a high-temperature oven for shape settings. Stents were cleaned by sonication in acetone, water, and 2-propanol followed by drying in a nitrogen stream. Surface activation was achieved by treatment with UV/ozone before use for amino-functionalization. Substrates were immersed in a solution of N-[3-(trimethoxysilyl)-propyl]ethylenediamine in dry toluene, and the desired amount of isocyanate end group terminated six-arm star-shaped copolymer of 80% ethylene oxide and 20% propylene oxide NCO-sP(EO-*stat*-PO) (M_n= 12,000g/mol; PD=1.15) was dissolved in dry tetrahydrofurane under an inert gas atmosphere. Thereafter, the solution was filtered through 0.2 µm syringe filters and used for hydrogel coating. For dip-coating, stents were placed in 300 µl polymer solution. For bio-functionalization, following solutions were prepared in PBS: 50 µg/mL RGD + 1µg/ml P-selectin or 50 µg/mL RGD + 1 µg/ml LL-37 + 1 µg/ml P-selectin. Five min after hydrogel coating, samples were incubated in corresponding solutions overnight, and washed thoroughly with millipore water. Stents were implanted through an incision into left carotid arteries of *Apoe*$^{-/-}$ mice by insertion into 250 µm silicon tubes, careful forward feeding of the stent whilst retracting the tube to allow for shape memory-based expansion. After 1-4 weeks, stents were harvested, fixed with 4% paraformaldehyde, and embedded in methylmethacrylate (Technovit 9100, Heraeus). Specimens were sliced and polished using a cutting-grinding system technique (Exakt 400CS) using a diamond-band saw (Exakt 300) resulting in 50 µm sections, and treated with H_2O_2 for 15 min and with Giemsa stain for 30 min. Images were recorded using 2-photon microcopy (23) or a Leica DMLB microscope (Leica, Wetzlar) using Diskus software. To assess re-endothelialization, 5 µm cryosections adjacent to the stented carotid

artery were stained with a monoclonal antibody to CD31 (Santa Cruz) including nuclear counter-staining. Luminal coverage with $CD31^+$ endothelial cells was measured using Diskus software (Hilgers, Germany). The presence of $CD31^+$ endothelial cells (red) in the luminal lining within the stented area of carotid arteries was assessed by immunofluoresence staining for CD31 and 2-photon microscopy of arteries prepared one week after stent insertion and reembedded in agarose for analysis.

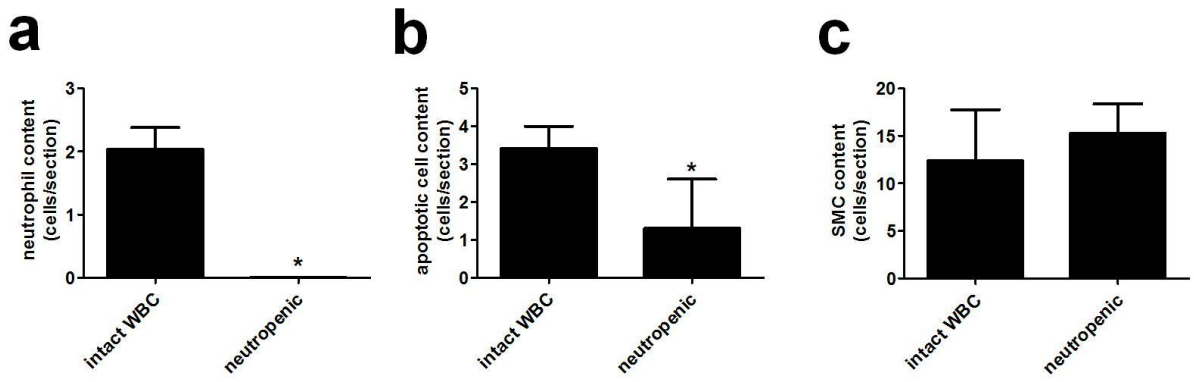

Figure S1: Neointimal cell composition. Immunohistochemical analysis of neointimal lesions one week after wire-injury in mice with intact white blood cell count (WBC) and in neutropenic mice. Displayed are $Ly6G^+$ neutrophils (a), $TUNEL^+$ apoptotic cells (b), and α-SMA^+ smooth muscle cells (SMCs) (c). n = 7-9. *$p<0.05$ versus intact WBC. Mann-Whitney test.

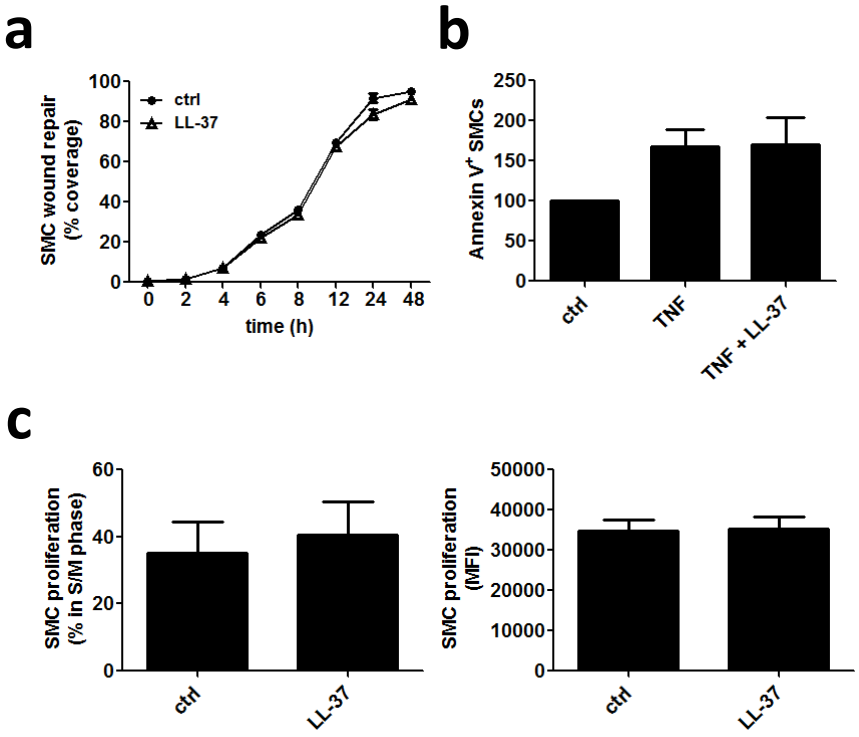

Figure S2: LL-37 does not affect migration, apoptosis, and proliferation of smooth muscle cells (SMCs). (a) Human aortic SMC monolayers were subjected to scratch injury and the recovered wound area was expressed as percentage of the initial wound area. Representative photomicrographs and quantified data are displayed. n = 5. **(b)** SMCs were treated with TNF (50 ng/ml) in presence or absence of LL-37 and apoptosis was quantified by flow cytometry after annexin-V staining. n = 4. Kruskal-Wallis-test with posthoc-Dunn-test. **(c)** SMC proliferation was assessed by cell cycle analysis using propidium iodide (left) or BrdU incorporation assays (right). Analyses were performed in cells treated with vehicle (ctrl) or LL-37. n = 5. Mann-Whitney test.

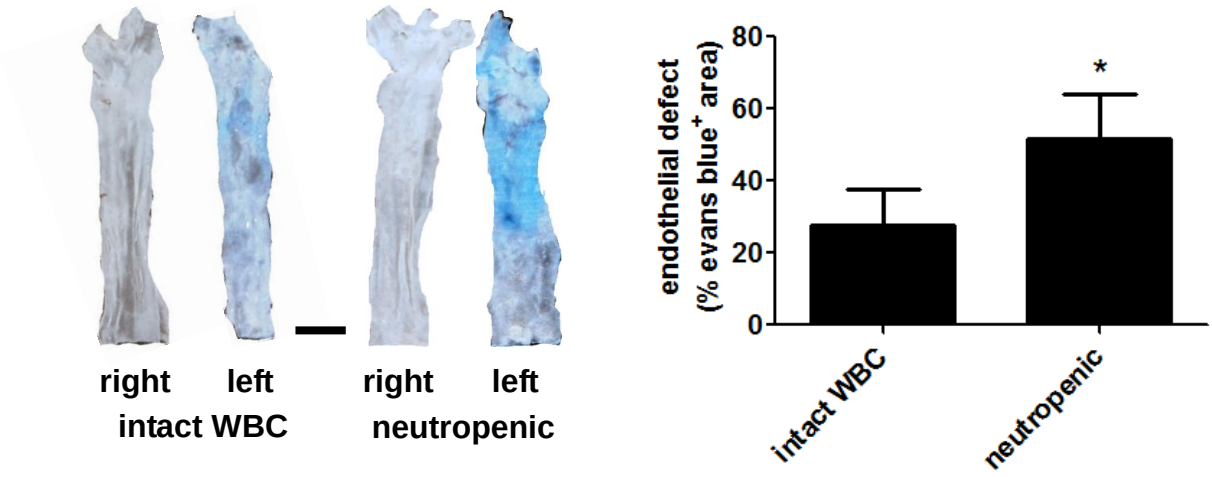

Figure S3: Neutropenia impairs endothelial recovery. Arterial injury was initiated in the left carotid artery of mice with intact white blood cell count (WBC) or neutropenia. After five days, mice were injected with Evans blue and the amount of Evans blue-positive area was assessed in *en face* preparations. Displayed are representative carotid arteries of mice with intact WBC (left) or neutropenia (middle). Scale bar represents 500 µm. Evans blue-positive area was quantified and expressed as % of total carotid area (right). n = 8. *$p<0.05$ versus intact WBC. Mann-Whitney test.

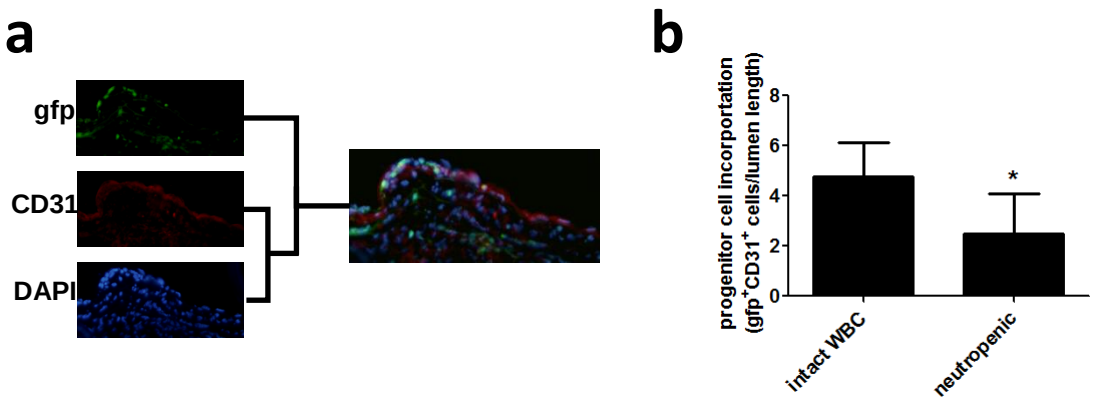

Figure S4: Neutropenia reduces incorporation of bone marrow-derived cells into the endothelial lining. *Apoe*$^{-/-}$ mice were reconstituted with gfp-fluorescent bone-marrow cells. Wire-injury was performed in neutropenic mice and in mice with intact white blood count (WBC). After one week, the integration of gfp^{+} cells into the endothelial lining was analyzed by immunohistochemistry. **(a)** Representative image of analysis of CD31^{+}gfp^{+} cells in serial sections. **(b)** Quantification of CD31^{+}gfp^{+} cells. n = 8. *p<0.05 versus intact WBC. Mann-Whitney test.

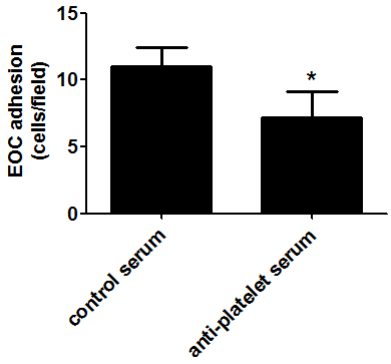

Figure S5: Platelet depletion reduces adhesion of EOCs. Adhesion of injected human EOCs to injured carotid arteries in mice treated with anti-platelet serum or control serum. Data are expressed as number of EOCs adherent to the lumen of the injured artery. n = 6. *p<0.05 versus control serum. Mann-Whitney test.

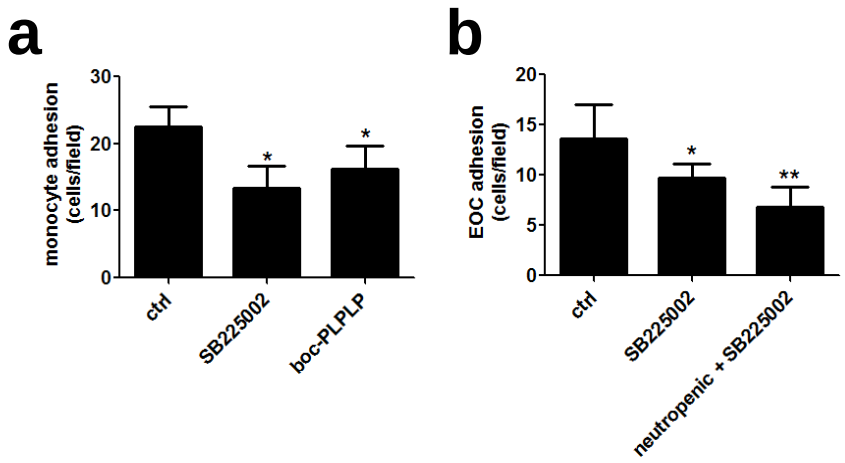

Figure S6: Adhesion of adoptively transferred monocytes and EOCs partially depends on CXCR2. (a) Adhesion of transferred human monocytes to injured carotid arteries following pre-treatment with vehicle (ctrl), a CXCR2 antagonist (SB225002, 100 µg/ml), or a FPR antagonist (boc-PLPLP, 10 µM). Data are expressed as cells adherent to the lumen of the injured artery. n = 6. *$p<0.05$ versus control group. **(b)** Adhesion of adoptively transferred human EOCs to injured carotid arteries following pre-treatment with vehicle (ctrl) or a CXCR2 antagonist (SB225002, 100 µg/ml). Neutropenia was induced by injection of anti-Ly6G antibody. Data are expressed as cells adherent to the lumen of the injured artery. n = 9. *$p<0.05$ versus control, **$p<0.05$ versus SB225002. Kruskal-Wallis-test with posthoc-Dunn-test.

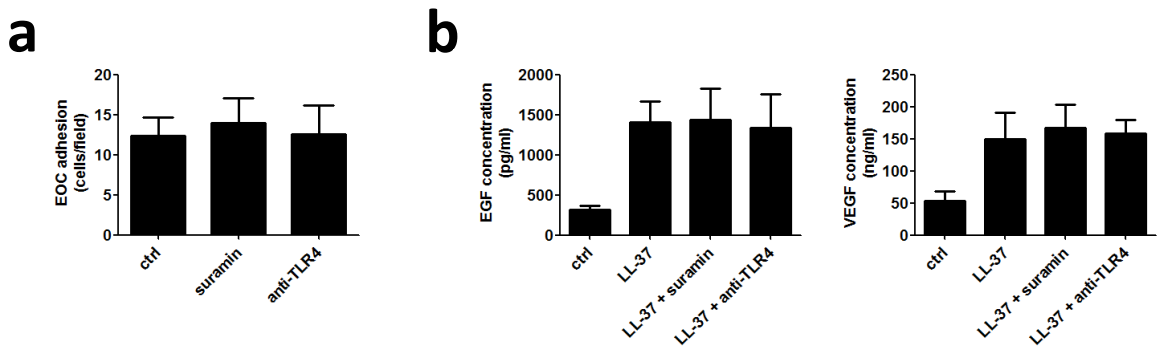

Figure S7: Role of P2 receptors and TLR4 in LL-37-mediated EOC activation. (**a**) Adhesion of injected human EOCs to injured carotid arteries following pre-treatment with vehicle (ctrl), suramin (100 µM) or a TLR4-neutralizing antibody (W7C11, 10 µg/ml). Data are expressed as EOCs adherent to the lumen of the injured artery. n = 6. (**b**) EOCs were treated with medium (ctrl) or LL-37 (1 µg/ml) in presence or absence of suramin (100 µM) or a TLR4-neutralizing antibody (W7C11, 10 µg/ml). n = 4. Kruskal-Wallis-test with posthoc-Dunn-test.

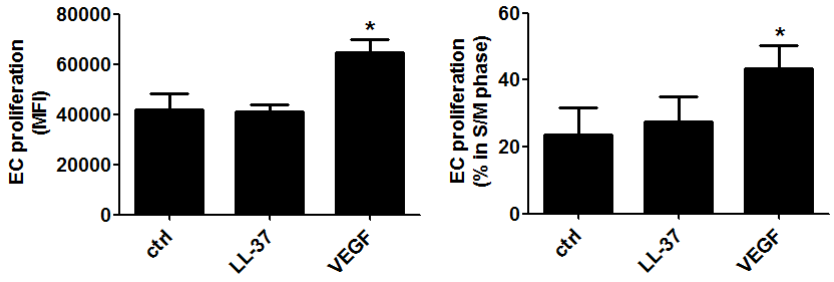

Figure S8: LL-37 does not induce endothelial cell (EC) proliferation. Human aortic ECs were treated with medium (ctrl), LL-37 (1 µg/ml, 24 h), or VEGF (20 ng/ml, 24 h) and proliferation was assessed by BrdU incorporation (left) or cell cycle analysis using propidium iodide (right). n = 5. *$p<0.05$ versus control and LL-37 treatment. Kruskal-Wallis-test with posthoc-Dunn-test.

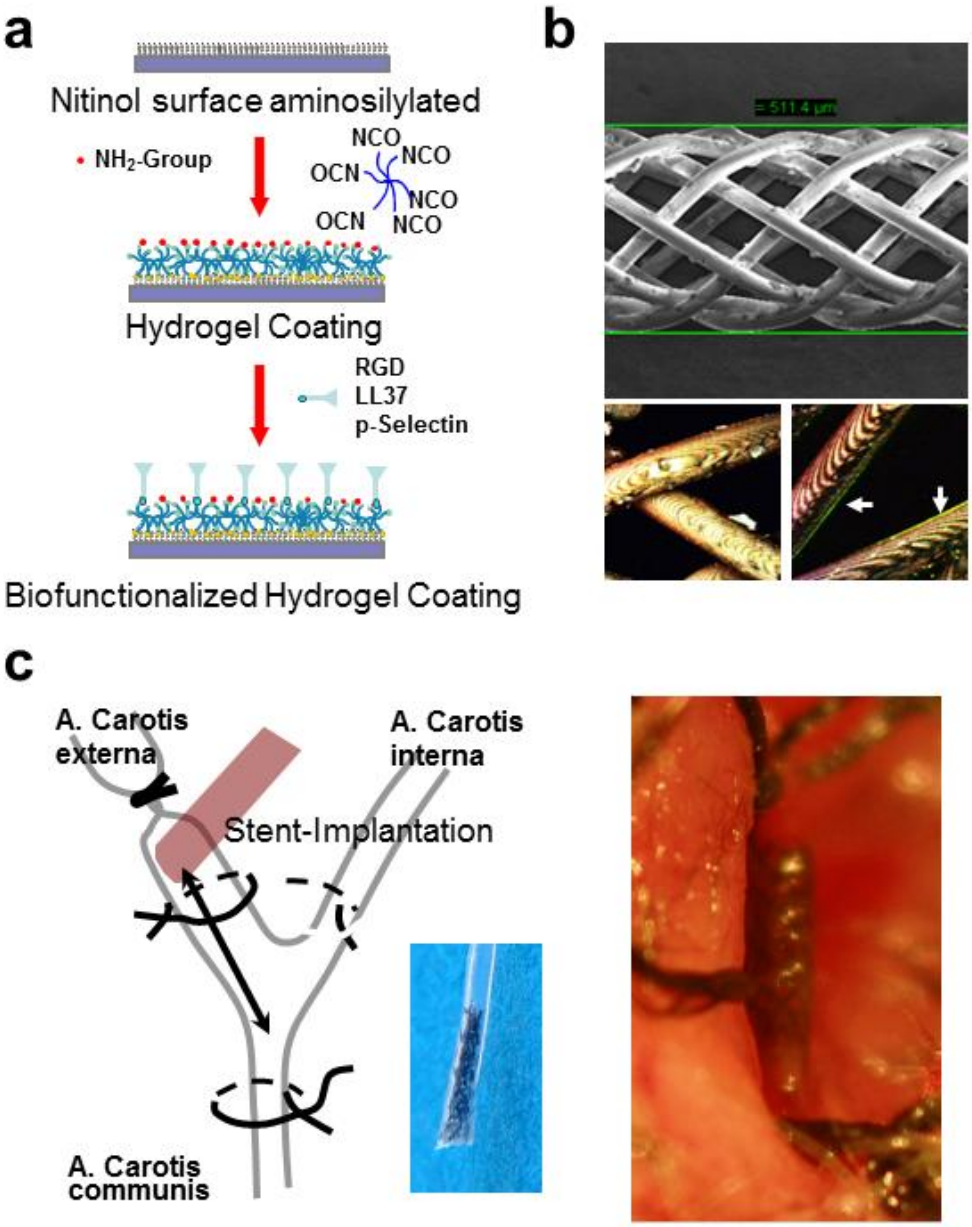

Figure S9: Development of an LL-37-coated Nitinol stent. (**a**) Schematic overview of Nitinol coating. Nitinol wires were aminosilanized with N-[3-trimethoxysilyl-propyl] ethylenediamine, coated with six-arm star-shaped polyethylene glycol, and biofunctionalized by covalently binding P-selectin and RGD-peptide or a combination of P-selectin, RGD-peptide, and LL-37. (**b**) Scanning electron microscopy image of a Nitinol stent (top). LL-37 coating was evidenced by use of green fluorescent LL-37 (bottom right, arrows) employing 2-photon microscopy. (**c**) Schematic overview of stent implantation procedure (left) and *in situ* image of implanted stent (right).

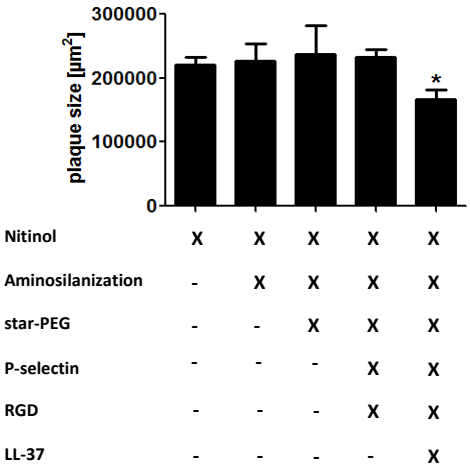

Figure S10: Neointima sizes are reduced in mice receiving LL-37-coated stents. Stents prepared as indicated were implanted into *Apoe*$^{-/-}$ mice and the neointimal area was analyzed one week later after Giemsa staining. n = 7-10. *p<0.05 versus all other groups. Kruskal-Wallis-test with posthoc-Dunn-test.

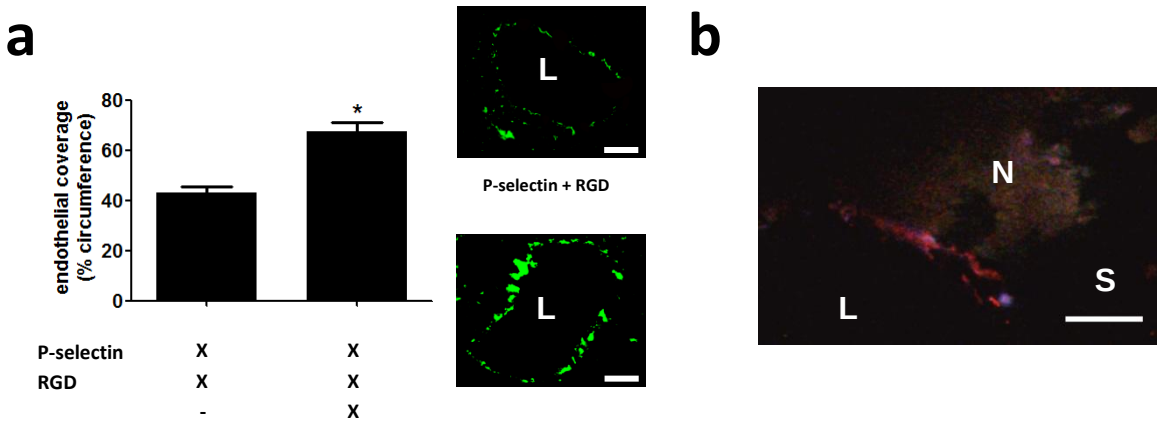

Figure S11: Reendothelialization is improved in arteries with LL-37-coated stents. A: Biofunctionalized stents coated as indicated were implanted into *Apoe*$^{-/-}$ mice and the luminal endothelial coverage was assessed by immunofluoresence staining for CD31 in cryosections directly adjacent to the proximal end of the stent after one week. n = 8. *p<0.05 versus P-selectin/RGD-coated stent. Mann-Whitney test. Scale bar, 25 µm. L, lumen. **B:** High abundance of CD31^{+} endothelial cells (red) in the luminal lining within the stented area of carotid arteries that had received LL-37-coated stents, as evidenced by immunofluorescence staining and 2-photon microscopy of agarose-embedded arteries prepared one week after stent insertion. Scale bar, 25 µm. L, lumen; N, neointima; S, stent strut.

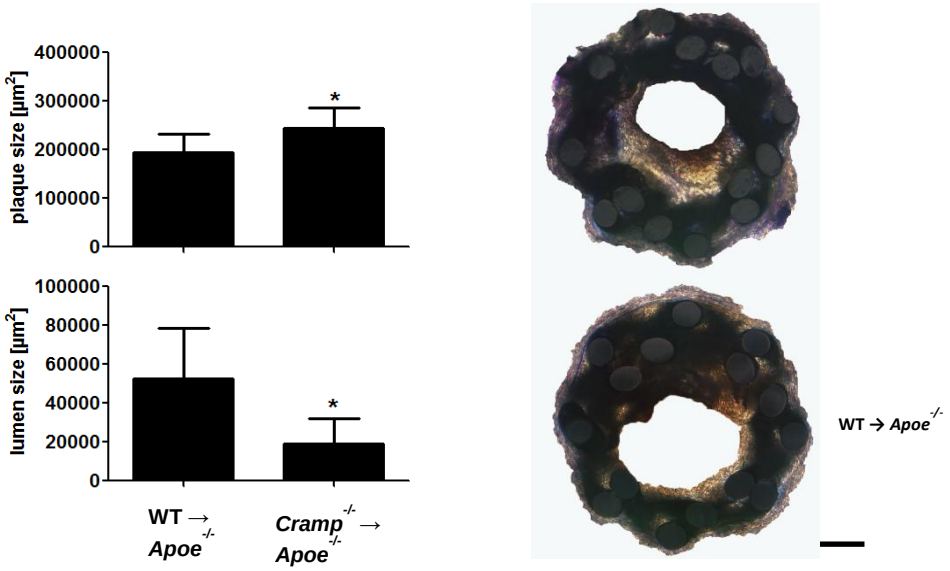

Figure S12: CRAMP prevents in-stent stenosis. *Apoe*$^{-/-}$ mice were reconstituted with bone marrow from C57Bl/6 mice (WT → *Apoe*$^{-/-}$) or from *Cramp*$^{-/-}$ mice (*Cramp*$^{-/-}$ → *Apoe*$^{-/-}$). Nitinol stents were implanted and based on Giemsa staining (right) neointimal area (top left) and lumen size (bottom left) were analyzed one week later. Scale bar, 100 µm. n = 8. *p<0.05 versus WT → *Apoe*$^{-/-}$. Mann-Whitney test.

Table S1: Differential leukocyte counts in mice with intact WBCs or neutropenia.
All values are given in cell count/ml venous blood.

	neutrophils	inflammatory monocytes	resident monocytes	T lymphocytes	B lymphocytes
Intact WBC	$8.1x10^5$ +/- $1.8x10^5$	$3.1x10^5$ +/- $0.7x10^5$	$1.9x10^5$ +/- $0.6x10^5$	$2.5x10^6$ +/- $0.6x10^6$	$1.9x10^6$ +/- $0.2x10^6$
neutropenic	$0.4x10^5$ +/- $0.1x10^5$	$3.3x10^5$ +/- $0.3x10^5$	$1.8x10^5$ +/- $0.8x10^5$	$2.7x10^6$ +/- $0.8x10^6$	$2.4x10^6$ +/- $0.6x10^6$
p-value	0.001	0.531	0.980	0.725	0.493

Table S2: Neointimal cell composition in mice with intact WBCs or neutropenia.

	total cells	SMC[a]	macrophages[b]	T lymphocytes[c]	neutrophils[d]	collagen[e]
Intact WBC	37.3 +/- 3.8	32.9 +/- 8.1	20.7 +/- 8.6	10.3 +/- 7.8	7.9 +/- 5.2	23.1 +/- 9.3
neutropenic	32.6 +/- 5.3	41.7 +/- 6.2	16.1 +/- 4.0	17.5 +/- 8.1	1.2 +/- 0.8	41.6 +/- 8.2
p-value	0.513	0.084	0.068	0.352	0.031	0.029

[a] data are displayed as α-SMA$^+$ cells in % of total cells
[b] data are displayed as Mac2$^+$ cells in % of total cells
[c] data are displayed as CD3$^+$ cells in % of total cells
[d] data are displayed as Ly6G$^+$ cells in % of total cells
[e] data are displayed as collagen$^+$ area in % of total neointimal area after Gomori trichrome stain

Table S3: Immunohistochemical analysis of SMC homeostasis 1 week after wire injury.

	Proliferating SMCs[a]	Apoptotic SMCs[b]
Intact WBC	1.2 +/- 0.9	0.8 +/- 0.7
neutropenic	0.6 +/- 0.4	0.7 +/- 0.3
p-value	0.718	0.913

[a] data are displayed as α-SMA^{+}KI-67^{+} cells per neointima
[b] data are displayed as α-SMA^{+}TUNEL^{+} cells per neointima

Table S4: Differential leukocyte counts in mice depleted of monocytes.

All values are given in cell counts/ml venous blood.

	neutrophils	inflammatory monocytes	resident monocytes	T lymphocytes	B lymphocytes
Monocyte-depleted	$9.8x10^5$ +/- $2.2x10^5$	$0.2x10^5$ +/- $0.1x10^5$	$0.1x10^5$ +/- $0.1x10^5$	$2.1x10^6$ +/- $0.4x10^6$	$2.6x10^6$ +/- $0.8x10^6$

Table S5: mCXCL1 and CXCL12 serum levels in mice with intact WBCs or neutropenia.
All values are given in pg/ml venous blood.

	mCXCL1 (KC)	CXCL12 (SDF-1α)
Intact WBC	107.3 +/- 20.2	20.1 +/- 8.3
neutropenic	112.7 +/- 25.6	18.7 +/- 9.7
p-value	0.782	0.851