Material and Methods

Isolation of murine and human neutrophils

To isolate and purify neutrophils from mouse bone marrow, femur and tibiae of mice were removed. The end of the bones were cut off and flushed with a syringe with 1% bovine serum albumin in phosphate buffered saline. The cell suspension was passed through a 70 µm cell strainer. After centrifugation (500 g, 10 min) the cell pellet was resuspended in 1% bovine serum albumin in phosphate buffered saline. The cells were then layered over a density gradient of Histopaque®-1119 and Histopaque®-1077 (Sigma-Aldrich) and centrifuged (700 g, 30 min, without break). After collecting the neutrophils at the interface of both histopaque layers cells were washed and counted. Purity of neutrophils was assessed by flow cytometry using antibodies from BD biosciences (CD45 (APC-Cy7; Clone 30-F11); CD11b (V500; Clone M1/70); Ly6G (V450; Clone 1A8)). Cell suspensions were analyzed using a FACS Canto II flow cytometer (BD, Biosciences) and data were analyzed by FlowJo Software (Treestar Inc.). Human neutrophils were isolated from venous blood of healthy volunteers. To obtain neutrophil cell suspensions, whole blood was diluted 1:1 with phosphate buffered saline and layed over density gradients and processed as described above.

Imaging Agent

Neutrophil Elastase FAST 680 (Perkin Elmer, Waltham, Massachusetts) is a preclinical fluorescence imaging agent emitting light in the near infrared spectrum (excitation 675 nm / emission 693 nm). It consists of a dedicated peptide sequence (PMAVVQSVP) with two VivoTag-S680 fluorochromes, which are self-quenched and become highly fluorescent after cleavage by neutrophil elastase. Plasma half-life of the agent is 3 hours with renal clearance only[1].

In vitro activation of Neutrophil Elastase 680 FAST

Experiments were carried out in 96-well plates with black sides and bottom in 100 µl/well at 37°C. For dose-response experiments 50,000 cells/ml were lysed with 0.01% Triton X and incubated with different concentrations (0.5 µM - 10 µM) of Neutrophil Elastase 680 FAST. For cell stimulation, 50,000 cells/ml were treated with phorbol-12-myristate 13-acetate (PMA)/Ionomycin (0.5 µg/ml or 1 µg/ml; Sigma-

Aldrich) and N-Formyl-Met-Leu-Phe (fMLP, 10 µg/ml or 1 µg/ml, Sigma-Aldrich) and incubated with 5 µM Neutrophil Elastase FAST 680. For blocking experiments 50,000 cells/ml were lysed with 0.01% Triton X and incubated with different concentrations of the selective elastase inhibitor, sivelestat [2] (S7198, Sigma-Aldrich, St.Louis, USA) ranging from 0.01 nM - 2000 nM for 30 min; thereafter the NIRF agent (5 µM) was added. The fluorescence intensity was measured over a time period of 90 min, and conducted at excitation/emission wavelengths of 663/690 nm using a fluorescence plate reader (TECAN infinite M1000 pro). To analyze probe cleavage kinetics, the Michaelis constant (K_m) was calculated using the Michaelis-Menten equation under consideration of determined reaction rates under different substrate concentrations, and IC_{50} values of sivelestat were determined.

Mouse model of atherosclerosis

LDL-receptor deficient mice (*$LDLr^{-/-}$,* B6.129S7-Ldlrtm1Her/J, obtained from the Jackson Laboratory) were used. At the age of 14 to 15 weeks *$LDLr^{-/-}$* mice were placed on a Western Type high fat diet (HFD, 21% fat, 0.15% cholesterol, 19.5% casein, Altromin, Lage, Germany) for a period of 4, 8 or 12 weeks before imaging by the FMT-XCT hybrid system. After imaging, mice were sacrificed for ex vivo analyses. All animal experiments were approved by local authorities (55.2.1.54-2532-115-13, Regierung von Oberbayern, München, Germany) in accordance with the German animal protection law.

In vivo imaging: Fluorescence Molecular Tomography-X-ray computed tomography (FMT-XCT)

For in vivo experiments, mice were imaged at 0 (n=10), 4 (n=10), 8 (n=8) and 12 (n=4) weeks of HFD. Before each imaging time point, the chest was shaved followed by chemically depilation as the fur is significantly absorbing and scattering the near-infrared fluorescence (NIRF) light. 100µL of Neutrophil Elastase 680 FAST was injected via tail vein injection (4 nmol/100µL). Imaging was performed at 4h post probe injection. Atherosclerotic mice that did not receive the imaging agent were used to determine the level of autofluorescence. To improve segmentation of the chest XCT images, an intravascular CT contrast agent (Exitron nano 12000, 100µl/25g mouse) was immediately injected before scanning. Mice were anesthetized by isoflurane inhalation (isoflurane 2.5 %, O_2 0.85 L/min) during the

imaging measurement. After finishing the experiments, mice were euthanized for further ex vivo analysis. All mice were imaged using a FMT-XCT hybrid imaging system [3, 4]. The system combines two imaging modalities, namely FMT and XCT, for imaging small animal disease models. Co-registered XCT images with high resolution can provide anatomical information for FMT, which improves the FMT three-dimensional functional and molecular reconstruction performance in a fundamental way [4]. For data acquisition, each mouse was first illuminated by a 680nm laser in a 360° trans-illumination mode. Excitation and emission images were both acquired at 20 equally spaced gantry locations by using a scientific charge-coupled device camera cooled at -80˚C with two different sets of filters placed in front of it (one for excitation and one for emission). Around 30 different positions of laser illumination in the region of interest were calculated automatically by first-acquired white light reference images at each gantry location. Furthermore, the mouse was scanned by using the integrated XCT system, which collected projections over a field of view of 360°. The current and energy of the x-ray tube was 450 µA and 80 kV. After FMT-XCT data acquisition, three-dimensional fluorophore distribution was reconstructed by using sparse linear equations and sparse least squares methods [5] and XCT anatomical information as prior.

Anatomical images were reconstructed by filtered back projection method and semi-automatically segmented based on the gray-scale slices. Different optical parameters (absorption coefficients and scattering coefficients) were assigned to different organs for every mouse. Then, FMT inversion was performed iteratively to locate the fluorescent distribution. To quantify and statistically analyze the FMT reconstruction, mean values of the region of interest of fluorescence intensities (around 5 slices, 1 mm interval) were calculated afterwards.

Whole-body Cryoslicing & Fluorescence Microscopy of Neutrophil Elastase FAST 680

After imaging, mice were sacrificed and whole-body cryoslicing was performed followed by fluorescence microscopy to investigate the localization of the fluorescence signal ex vivo. This method provides slice images in the same orientation (transverse plane) as FMT-XCT data acquisition showing anatomy and near-infrared reagent distribution. After each time point, mice (0 weeks of HFD, n=2; 4, 8, and 12 weeks of HFD, n= 3) were sacrificed and frozen at -80°C. For

cryoslicing, the mouse torso was embedded in a mixture of O.C.T. (Optimal Cutting Temperature) medium and India Ink. Cryoslice imaging of the mice was performed using a multispectral imaging system combined with a cryomicrotome (Leica) [6]. Transversal slices of the thorax 150 µm apart were acquired. After each slice a planar colored image and planar fluorescence image (excitation 680 nm) were taken using a filtered white light source and a sensitive charge-coupled camera. For detailed further analyses, cryoslices (20 µm thickness) of the ascending aorta and the aortic arch were placed on adhesive-glass slides (SuperFrost plus, Thermo Scientific), cell nuclei were counterstained with DAPI and embedded in ProLong Gold (ThermoScientific). Sections were imaged by fluorescence microscopy using appropriate filters (Cy5.5 filter and DAPI) or brightfield (Zeiss Axio Imager 2, Zeiss Zen analysis software). Additional 5 µm cryosections through the aortic root were obtained and incubated with Neutrophil Elastase FAST 680 for 15 minutes. Sections were washed and mounted using Vectashield with DAPI (Vector Laboratories), and immediately visualized using a Leica TCS SP5 confocal microscope (Leica Microsystems).

Immunofluorescence Staining and Histological Analysis

Immunofluorescense staining of neutrophils was performed using 5 µm cryosections through the aortic root. After fixation using 4% PFA (1 hour), slides were blocked (1% bovine serum albumin, 2% mouse, rabbit and horse serum, 0.1% TX100 in PBS; Sigma Aldrich), and incubated with primary anti-Ly6G antibody (rat-anti mouse Ly6G, Clone 1A8, 1:200 dilution, BD), and secondary detection performed using anti-rat Alexa Fluor 488-conjugated goat-anti-rat antibody. Sections were coverslipped using Vectorshield mounint medium with DAPI. The extent of atherosclerosis and neutrophil counts in atherosclerotic lesions were evaluated by immunohistological methods. After in vivo imaging, mice (4, 8, and 12 weeks of HFD, n= 3) were euthanized and the heart and aortic arch were perfusion-fixed with phosphate buffered saline and 4% formalin. After the dehydrating process the aortic root and aortic arch was embedded in paraffin. Sectioned in the transverse plane (2.5 µm thickness, 25 µm apart) were placed on adhesive-glass slides, and neutrophil staining was performed using anti-Ly6G antibody (rat-anti mouse Ly6G, Clone 1A8, 1:200 dilution, BD). Following primary antibody incubation, neutrophils were visualized with the Dako Labeled Streptavidin-Biotin System, Horseradish

Peroxidase (LSAB System, HRP). A nuclei counterstain was performed with haematoxylin. The slides were analyzed by counting the number of neutrophils using a brightfield microscope (Leica Leica DM 4000 B) and measuring the plaque size using ImageJ software.

Statistics

Data are represented as mean ± SD. Comparisons over time were analyzed by one-way Analysis of Variance (ANOVA) followed by Bonferroni post-tests for multiple comparisons using GraphPad Prism Software 6.0. Differences with p-values < 0.05 were considered to be statistically significant.

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