Endotoxinemia accelerates atherosclerosis via electrostatic charge-mediated monocyte adhesion

Schumski: Histones promote atherosclerosis in endotoxinemia

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- SUPPLEMENTAL MATERIAL -

Supplemental Methods

Flow cytometry

Leukocyte counts were quantified by flow cytometry. Staining of single cell suspensions of blood and bone marrow were conducted using combinations of antibodies specific for CD45 (A20, eBioscience), CD11b (M1/70, eBioscience), CD115 (AFS98, eBioscience), Gr1 (RB6-8C5, BioLegend). Before cell staining, red blood cell lysis was performed. Furthermore, aortas were digested by liberase (1.25mg/ml, Roche) and single cells were labeled with antibodies to CD11b (M1/70, bioLegend), Ly6G (1A8, BioLegend), MHC II (M5/114.15.2, BD Bioscience), Gr1 (RB6-8C5, BioLegend), F4/80 (BM8, BioLegend), CD45 (A20, eBioscience), slan (M-DC8, Miltenyi biotec). Cells were washed with Hanks Balanced Salt Solution (HBSS) and directly analyzed by flow cytometry using a FACSCanto II (BD). Absolute cell numbers were assessed by use of CountBright™ absolute counting beads (Invitrogen). Data were analyzed with FlowJo Software (10.1 Flowjo LLC).

Cell free DNA measurement

Cell free DNA was measured in the plasma with Quant-iT PicoGreen dsDNA assay (Life Technologies) the mean fluorescence intensity was quantified and according to the standard curve, results are expressed as ng/ml. Samples were measured with a plate reader (Tecan infinite F200 pro).

NET-ELISA

Neutrophil extracellular traps were measured in plasma by detecting myeloperoxidase and cell free DNA as described elsewhere[49]. Briefly, MPO-DNA complexes were quantified by modified MPO ELISA (Hycult Biotech) combined with cell death detection kit (Roche).

Endotoxin measurement

LPS plasma levels were assessed with Pierce Chromogenic Endotoxin Quant kit (Thermo Fisher Scientific). Plasma samples were diluted 1:50 in endotoxin free water and heat inactivated for 15 min at

70°C. Amebocyte lysate reagent was added to each well, incubated for 30 min followed by chromogenic substrate solution incubation. After 6 min of incubation, the stop solution was added, and the absorption was measured with Tecan infinite F200 pro (OD 405 nm).

Histology and Immunohistochemistry

The size of atherosclerotic plaques was measured in aortic roots after staining with Hematoxylin (Merck) and Eosin (Roth) followed by computerized image analysis and quantification (ImageJ, 1.48v). Aortic roots were stained with antibodies to Ly6G (1A8, BD Biosciences) and Mac2 (M3/38, biozol). Nuclei were counter-stained with DAPI (Thermo Fischer). A Leica DM4000 microscope with a 20x objective (Leica Microsystems) and a Leica DFC 365FX camera were used to capture images.

In vitro adhesion assay

Blood was drawn from healthy volunteers. Human neutrophils were isolated by polymorphprep according to the manufacturer's instructions. Classical monocytes were isolated via negative selection using monocyte isolation kit II (MACS Miltenyi biotec) and T-cells were isolated using the CD4+ T cell Isolation Kit (Miltenyi biotec). Adhesion of monocytes to NETs was studied under static and flow conditions. Isolated monocytes were labeled with CellTrace™ calcein violet AM (Thermo fisher scientific). Monocytes were added ($0.5x10^5$ cell/well) to flat bottom 96-well plates (Falcon corning) or flow chambers (µ-slide $VI^{0.4}$ ibiTreat (Ibidi)) containing either neutrophils ($2x10^5$ cells/well, labeled with cell tracker red CMTPX (Thermo fisher scientific)) or NETing neutrophils (25 µM calcium ionophore A23187 (Sigma Aldrich)) for 15 min or 3 min under applied flow (0.5 dyne/ cm^2; PHD ULTRA syringe pump, Harvard Apparatus). Non-adherent monocytes were washed off. Statically adherent monocytes were quantified in a microplate reader (Tecan infinite™ 200 pro). Monocytes adherent under flow conditions were counted manually. For each flow channel three fields were quantified and averaged. NETs were visualized by co-staining DNA with DAPI and citrullinated H3 or Sytox green nucleic acid stain (Thermo Fisher Scientific). To study monocyte adhesion to NETs, traps were treated with DNAse

(10U, Sigma Aldrich), antibodies to Histone H2a (10 µg/ml; Cell Signaling), Myeloperoxidase (10 µg/ml; AB1224, Merck), Proteinase 3 (10 µg/ml; MAB6134, R&D systems), Neutrophil elastase (10 µg/ml; Biorbyt), Cathepsin G (10 µg/ml; Biorbyt), LL-37 (10 µg/ml; Santa Cruz Biotechnology), S100A8/A9 (10µg/ml; A15105B, Biolegend), α-defensin (10 µg/ml; Hycult Biotech), CHIP (200 µg/ml, Pepscan). In another experimental setting monocytes were pre-incubated with antagonists to CCR1 (1 µM; BX471, Tocris), CCR2 (1 µM; RS504393, Tocris), CCR5 (1 µM; SB32437, Tocris), CCR5 (0.1 µM; DAPTA, Tocris), CXCR2 (1 µM; SB225002, Tocris), CXCR4 (1 µM; AMD3100, Sigma Aldrich), FPR1 (10 µM: Cyclosporin H, Tocris), FPR2 (10 µM; WRW4, Tocris), TLR1-2 (100 µM; CU CPT 22, Tocris), TLR4 (100 µM; C34, Tocris), TLR9 (100 µM; Hydroxychloroquine sulfate, Tocris), VLA-4 (10 µM; BIO1211, Tocris), P2x7 (0.1 µM; A 74 0003, Tocris), or P2y2 (10 µM; AR-C118925xx, Tocris). To block chemokine receptor signaling, cells were pretreated with Pertussis toxin (0.8 µg/ml, Sigma-Aldrich); Ca^{2+} mobilization was abrogated using the calcium chelator BAPTA AM (2 µM; Thermo Fisher Scientific). Finally, antibodies to Mac1 (M1/70, 10 µg, Biolegend) or LFA1 (10 µg, R7.1, Biolegend) were used to test the relevance of integrin activation.

Adhesion assay on endothelial cells

Plates and slides where coated overnight 4°C with 40 µg/ml collagen type I (ibidi). The next day 30,000 endothelial cells (SVECs) were seeded into these 96-well plates or into the µ-Slides (ibidi $VI^{0.4}$, ibiTreat) and grown to confluence. Before starting the experiment, SVECs where stimulated TNF (4h, 10 ng/ml). The stimulus was washed off and the endothelial cells where fixed with 1:1 CellCover (Anacyte) mixed with full medium for one hour. In the meanwhile, human neutrophils where stimulated with 25 µM A23187 (Sigma Aldrich) for 10 min and washed before adding 200,000 neutrophils per well/channel. Isolated classical monocytes were labeled with CellTrace™ calcein violet AM (Thermo fisher scientific). Monocytes were added ($0.5x10^5$ cell/well) to flat bottom 96-well plates (Falcon corning) or flow chambers (µ-slide $VI^{0.4}$ ibiTreat (Ibidi)) and left for adherence for either 15 min in static conditions or perfused for

3 min and washed off afterwards. Monocyte adhesion was recorded by fluorescence microscopy and analyzed offline.

Molecular Dynamics (MD) Simulations and Peptide Design

Three-dimensional (3D) structures of protein-protein complexes represent a good starting point for rationally design and develop bioactive compounds to modulate protein-protein interactions as demonstrated by our previous work[25,26,50]. Thus, the complex between human BRPF1 bromodomain and histone H2a peptide (PDB code: 4QYL) was selected and utilized to design potential histone H2a peptide inhibitors. The loop regions (residue 31-43 and 78-91) of bromodomain binding with the N-terminal tail of histone H2a were extracted and used as a starting peptide which were then docked onto the N-termini histone H2a (chain C of the nucleosome structure, PDB code: 1KX5). Based on the derived complex between the template peptide and histone H2a several peptides were rationally designed and then docked onto the N-terminal tail of histone H2a as well by application of protein-protein docking program (HADDOCK2.2 webserver[51]). The derived histone H2a -peptide complexes were subsequently subjected to structural optimization and binding free energy calculations to predict binding strength of peptides with histone H2a. Similar protocols and standard parameters as in our previous work were applied for these purposes[25,26]. Briefly, energy minimization was carried out for 10,000 steps (5,000 steps of steepest descent followed by 5,000 steps of conjugate gradient algorithm) and subsequently a short MD simulation (500 ps) was performed by using TIP3P water model and AMBER14SB force field for peptides and protein (histone H2a) and setting essential parameters at the standard values (e.g. temperature at 300 K, pressure at 1 bar, time step at 2 fs with SHAKE constraint). Binding free energy was calculated by using molecular mechanics/generalized Born surface area (MM/GBSA) approach (generalized Born model 8 with default parameters). MD simulations and binding calculations were carried out by using AMBER16 program. Binding affinity of the designed peptides were prioritized according to their predicted binding free energy and the peptide with the lowest binding free energy, which is CHIP (H-

CEPLSEVEDYLDSSLKYNAKDTINYC-OH containing S-S bond between Cys at the N- and C-terminus), was selected for synthesis and further experimental testing.

Zeta (ζ) potential

Cell surface charge of either untreated monocytes, monocytes incubated with sodium cholesterol sulfate (200 µM, Sigma-Aldrich) and sulfatase inhibitor STX64 (1 µM, Sigma-Aldrich), or monocytes incubated with oleylamine (200 µM, Sigma-Aldrich) was measured using a Malvern Zetasizer Nano. For ζ potential measurements, 10 µl cell suspension ($1x10^6$ cells/ml) was with mixed with 90 µl 300 mM sucrose (Sigma-Aldrich) and the sample was loaded at the bottom of a DTS1070 cuvette (Malvern Instruments) that was prefilled with 10 mM sodium chloride (Sigma-Aldrich). Measurements 30 runs per measurement, monomodal mode) were recorded at 37 °C as technical triplicates.

Atomic force microscopy

Neutrophils (0.5×10^6) were seeded on a flouro dish (WPI Inc.) with a glass bottom and left for adherence and NET-formation as described above. A single monocyte was captured on a cantilever (MLCT-010, Bruker) coated with 0.1 mg/ml concavalin A (Merck). Before capturing monocytes, the dish and cantilever were washed 3x (HBSS, Gibco) and the cantilever was routinely calibrated on a clean area in the probing dish. Monocyte viability was controlled by propidiumiodid (5 µl/sample, eBioscience) staining. AFM force spectroscopy was only performed with living cells (at least 8 cells per experimental setup). The single monocyte on the cantilever tip was brought above a single NET-structure and probed with an approach and retraction speed of 10 µm/s, the pulling range was set to 25 µm, and the maximal contact force applied to the monocyte was 200 pN. The monocytes were probed on an area of 10 μm^2 resulting in ten acquired force curves. Data were analyzed by using JPK Data Pocessing software (Version spm-5.0.96).

Monocyte-histone H2a binding assay

Isolated monocytes were incubated with 10µg of human biotinylated recombinant H2a. During incubation cells were chilled on ice and incubated with histone H2a for 15 min. Afterwards cells were washed and fixed with 4% PFA for 10 min and washed again. To detect bound histone H2a the recombinant protein was stained with straptaviden APC (biolegend) for 15 min and washed again. The adherent histone H2a was analyzed using either confocal microscopy or flow cytometry.

Sample preparation for MS

Isolated human neutrophils were stimulated to produce NETs. Alu I was added to each well to cut the NETs DNA into smaller soluble pieces. Samples were centrifuged to eliminate cell debris and keep the supernatant containing the DNA and associated NET proteins in solution. A commercially-available mass spectrometry sample preparation kit was utilized to ensure minimal sample loss and reproducibility: iST Kit for proteomic sample preparation (PreOmics GmbH). The preparation involved denaturation, alkylation, digestion and peptide purification. Denaturation, reduction of disulfide bridges, and cysteine alkylation was performed at 95°C for 10 min using 50ul of the iST Kit "LYSE" buffer. After a 5 min cooling step at room temperature, proteins were propionylated with 20ul of 2.5% propionic anhydride (Sigma) in ammonium bicarbonate (pH 7.5) at 50°C for 30 min and speed vacuumed until residual buffer had evaporated. Samples were transferred to the iST Kit cartridges, where digestion was carried out with Trypsin and LysC at a ratio of 1:100 ug of enzyme to protein and incubated at 37°C for 1 h at 500 rpm. Digestion was halted using 100 ul of the iST Kit "STOP" solution incubated for 1 min at room temperature and 500 rpm. Cartridges were then washed twice with 200 ul of the kit "WASH" solutions and eluted with 200 ul of 80%ACN + 0.25% TFA solution. The eluent was speed vacuumed until dryness and stored until MS analysis.

LC-MS/MS analysis

Peptides were re-suspended in 17 µl of 0.1% TFA. A total of 5.0 µl were injected into a nano-HPLC device (Thermo Fisher Scientific) using a gradient from 4% B to 90% B (solvent A 0.1% FA in water,

solvent B 80% ACN, 0.1% FA in water) over 90 min at a flow rate of 300 nl/min in a C18 UHPCL column (Thermo Fisher Scientific). Data was acquired in DDA positive mode using a Q Exactive HF spectrometer (Thermo Fisher Scientific). MS1 spectra were acquired at 60K resolution and AGC target value of 3e6 within a m/z range of 250 to 1600. The top 10 precursor ions were isolated with a window of 2.0 m/z and fragmented with NCE of 27 eV. Dynamic exclusion windows of 12.0 ppm and 20 s were used. MS2 spectra were obtained at 15K resolution and AGC target value of 1e5 within a m/z range of 200 to 2000.

MS data analysis

Raw files were searched against the whole human proteome sequences using MaxQuant 1.5.0 and the following parameters. Fixed modifications: Carbamidomethyl (C). Variable modifications: Acetyl (K); Dimethyl (KR); Methyl (KR); Methyl-Propionyl (K); Oxidation (M); Propionyl (K); Trimethyl (K) and Citrullination (R). Precursor mass tolerance: 4.5 ppm. Fragment mass tolerance: 20 ppm. Match between runs with default values. FDR thresholds of 0.01% for PSM, Protein and Site matches. The identified proteins were mapped to the String database (version 11.0) to retrieve their confidence interaction scores. Proteins were then visualized as a network in Cytoscape v3.4.0 and clustered by their confidence interactions scores. The Msms counts obtained from MaxQuant were used for node size, whereas for the node color, a continuous mapping of the proteins isoelectric points was used.

Supplemental Tables

Characteristic	Septic Patients	Characteristic	Control Patients
Age (years)	N=30	**Age (years)**	N=30
Median Sd	68.5 (42-93) 13.81	Median sd	66.5 (43-81) 10.058
Gender, n (%)		**Gender, n (%)**	
Female	14 (47)	Female	15(50)
Male	16 (53)	Male	15(50)
Race, n (%)		**Race, n (%)**	
Asian/Pacific Islander	1 (3)	Asian/Pacific Islander	1 (3)
Black, non-Hispanic	1 (3)	Black, non-Hispanic	3 (10)
White, non-Hispanic origin	27 (90)	White, non-Hispanic origin	19 (63)
Other	1 (3)	Other	7 (23)
Ethnicity, n (%)		**Ethnicity, n (%)**	
Not Hispanic or Latino	29 (97)	Not Hispanic or Latino	25 (83)
Hispanic or Latino	1 (3)	Hispanic or Latino	5 (17)
Cardiovascular parameters, n (%)		**Cardiovascular parameters, n (%)**	
Hypertension	30 (100)	Hypertension	28 (93)
Use of antihypertensive medication	24 (80)	Use of antihypertensive medication	24 (80)
Hypercholesterolemia	24 (80)	Hypercholesterolemia	23 (77)
Use of statins	17 (57)	Use of statins	19 (63)
Critical care unit, n (%)	24 (80)	**Critical care unit, n (%)**	24 (80)
Medical floor unit, n (%)	6 (20)	**Medical floor unit, n (%)**	6 (20)
Source of infection in Gram negative bacteremia (%)	**N=30**	**Negative blood cultures**	N=30

Bacteremia	8 (27)		
Genito-urinary tract	11 (37)		
Gastrointestinal	7 (23)		
Biliary	1 (3)		
Hepatic	1 (3)		
Lung	5 (17)		
Bone	1 (3)		
Skin	2 (7)		
Endocarditis	1 (3)		
Unknown	1 (3)		

Table I: Patient characteristics

Name	isoelectric point (pH)	Peptide Intensity
CTSG	11,37	39969000
HIST1H4F	11,36	29398000
HIST3H3	11,13	14526000
HIST1H2AC	11,05	1476600000
HIST1H1E	11,03	61022000
HIST1H1C	10,94	78378000
HIST1H1B	10,91	212620000
HIST2H2AC	10,9	31278000
HIST1H2AJ	10,88	154580000
HIST2H2AB	10,88	19278000
RNASE3	10,47	188140000
HIST1H2BH	10,32	64457000
HMGN2	10	85230000
MNDA	9,76	8253900
MPO	9,31	116350000
LYZ	9,28	111010000
RNASE2	9,2	51676000
LCN2	9,02	3092100
GAPDH	8,58	6349100
PFN1	8,47	45764000
GPI	8,44	10659000
ALDOA	8,39	33023000
PGK1	8,3	29612000
CFL1	8,26	23691000
HBD	7,97	3385500
PKM	7,95	18223000
PPIA	7,68	30168000
HMGB2	7,62	11583000
TKT	7,58	4874000
ENO1	6,99	59702000
CAT	6,95	26725000
ARPC2	6,84	5717200
HBB	6,81	514240000
ANXA1	6,64	7877000
UBA52	6,56	8612800
CDA	6,55	24939000
S100A8	6,5	447470000
UBE3C	6,27	7965800
CORO1A	6,25	74682000
MSN	6,09	19809000

SERPINB1	5,9	31708000
CAPG	5,82	10757000
S100A12	5,8	159940000
LTA4H	5,8	4206500
HNRNPU	5,76	6622400
S100A9	5,71	2873100000
FLNA	5,7	8698500
ALB	5,67	3494000
MYH9	5,5	119820000
VCL	5,5	1799800
HSPA1B	5,48	19043000
HSPA8	5,37	6078500
ACTG1	5,31	604210000
LCP1	5,29	4041800
ACTN1	5,25	11513000
AP2B1	5,22	5943500
ARHGDIB	5,08	269340000
VIM	5,05	32712000
TMSB4X	5,02	363210000
MYL1	4,97	5263400
SH3BGRL3	4,82	43684000
YWHAH	4,76	10081000
S100P	4,75	2945000
HDGF	4,7	1664200
MYL12A	4,65	21788000
CALM3	4,09	27758000

Table II: Proteomic analysis of NETs.

Supplemental Figures and Figure Legends

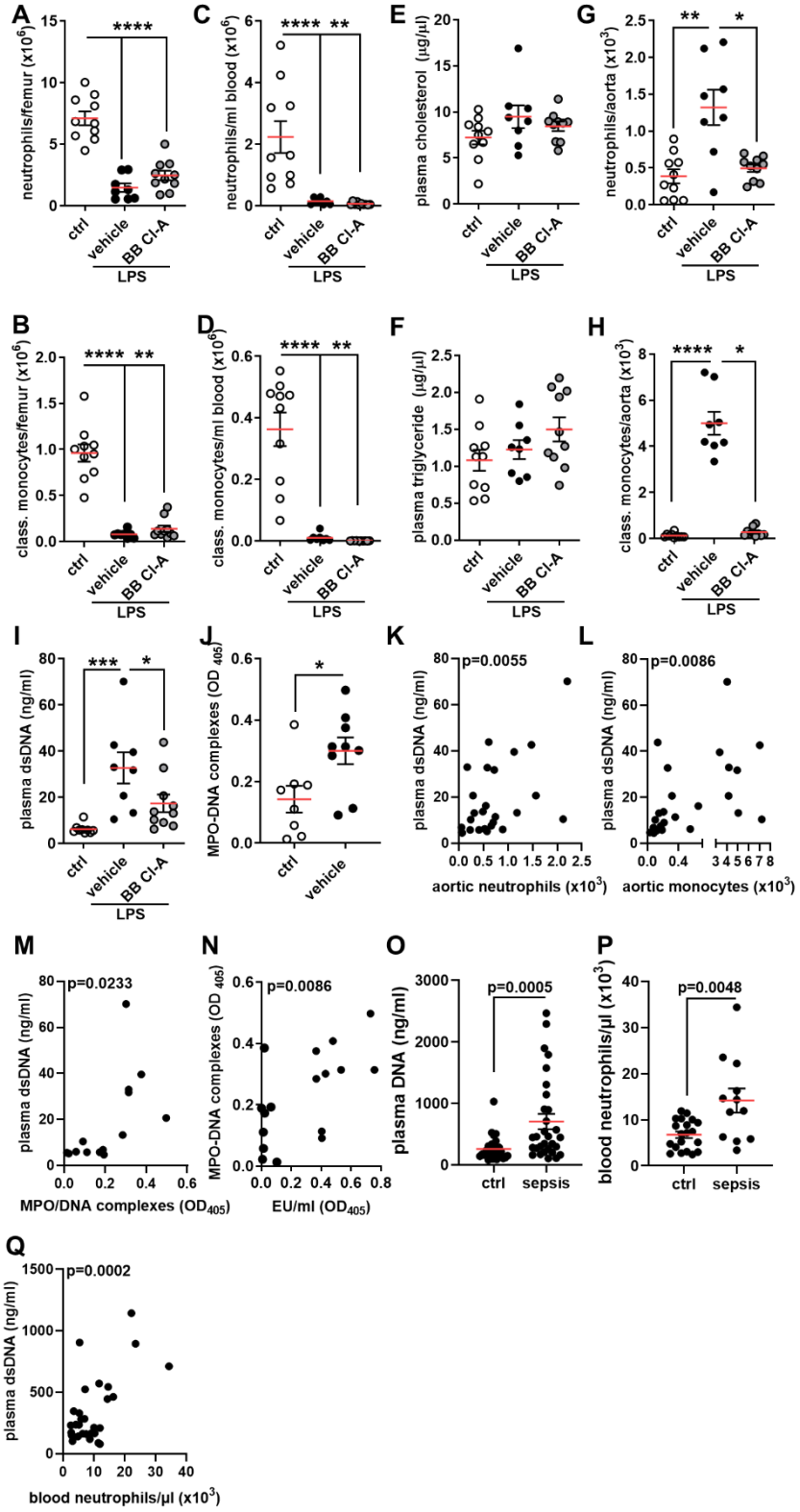

Figure I: Endotoxinemia-triggered NET release stimulates arterial myeloid cell recruitment. *Apoe*$^{-/-}$ mice were fed a HFD for 4 weeks and treated with either PBS (ctrl) or with LPS (1mg/kg BW). Another LPS-treated group received a BB Cl-amidine (BB Cl-A, 1mg/kg BW) 12 h before and along with LPS injection. **(A-D)** Flow cytometric quantification of neutrophils (A/C) and monocytes (B/D) in bone marrow (A/B) or blood (C/D). **(E/F)** Quantification of plasma cholesterol (E) and triglycerides (F). **(G/H)** Assessment of aortic neutrophil (G) and classical monocyte counts (H). **(I/J)** Quantification of cell free DNA in plasma (I) and circulating MPO-DNA complexes (J) as measure of NET release. **(K-N)** Pearson correlation of plasma dsDNA with aortic neutrophil counts (K), plasma dsDNA with aortic monocytes (L), plasma dsDNA with plasma MPO-DNA complexes (M), and plasma MPO-DNA complexes with plasma endotoxin units (N). **(O-Q)** Plasma dsDNA (O) quantified in patients with Gram-negative rod bacteremia or sepsis and with a cardiovascular risk profile. Displayed are also circulating neutrophil counts from the same patients (P). Please note that neutrophil counts were not available from all patients. Pearson correlation of plasma dsDNA and circulating neutrophil counts (Q). Assessed were samples from patients with confirmed Gram-negative sepsis or bacteremia and matched control patients. Data are analyzed by one-way ANOVA with Dunnett's multiple comparisons test (A, E, F, G, I), Kruskal-Wallis test with Dunn's post test (B-D, H), unpaired t-test (J), or Mann-Whitney test (O, P); *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. All data are presented as mean ±SEM.

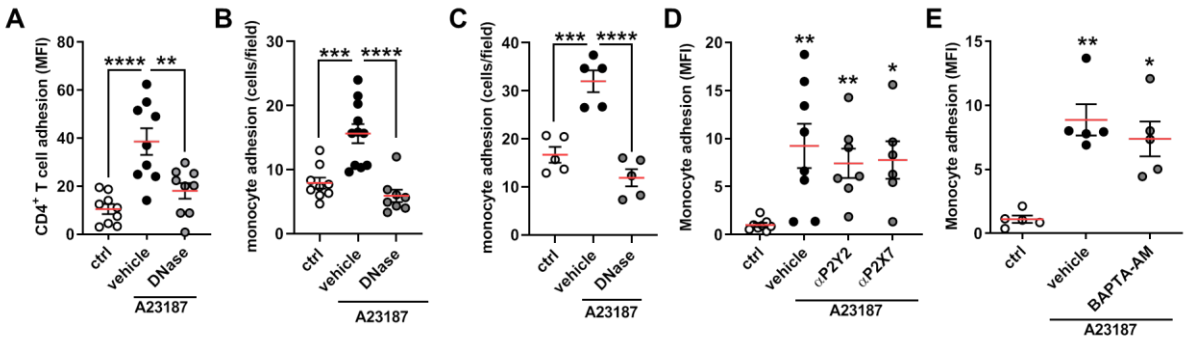

Figure II: Characterization of NET-driven leukocyte adhesion. (A) $CD4^+$ T cells were added to neutrophils (ctrl) or NETting neutrophils (induced by A23187) and adhesion was quantified by a fluorescence plate reader. NETs were also degraded by DNase I. **(B/C)** Endothelial cells were grown to confluence, activated with TNF (10 ng/ml, 4h) and A23187-activated neutrophils (25 µM, 10 min) were added on top to induce NET release. 1 hour after classical monocytes were sedimented (B) or perfused (C) and adhesion was quantified by fluorescence microscopy. **(D/E)** Monocytes were added to neutrophils (ctrl) or NETting neutrophils (induced by A23187) and adhesion was quantified by a fluorescence plate reader. Monocytes were pre-incubated with antagonists to purinergic receptors (A) or with cell permeant chelator (B). Data are analyzed by one-way ANOVA with Dunnett's multiple comparisons test (A-C) or by Kurskal-Wallis test with Dunn's post test (D, E); *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. All data are presented as mean ±SEM.

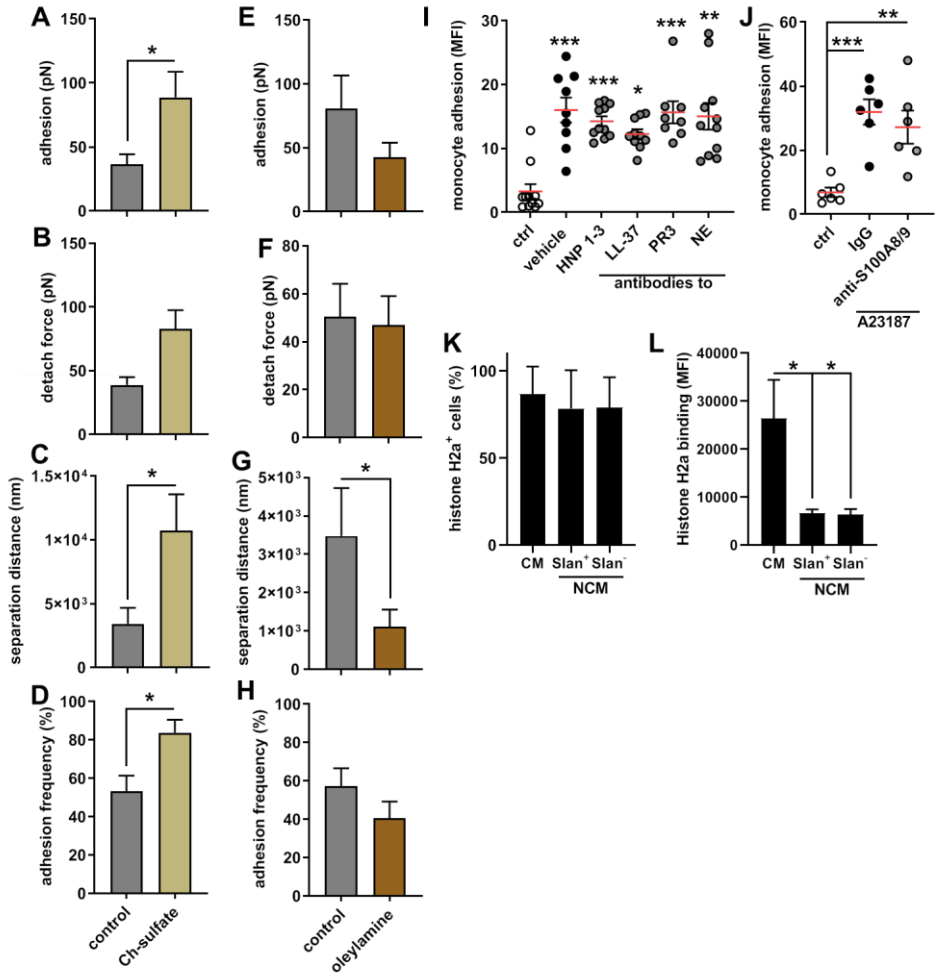

Figure III: Biophysical characterization of monocyte adhesion to NETs. (A-H) Atomic force microscopy analysis of adhesion of monocytes treated with cholesterol sulfate (Ch-sulfate) (A-D) or oleylamine (E-H) to NETs. Quantified are maximum adhesion force (A/E), detachment force while breaking the very last cell-to-NET bond (B/F), detachment distance (C/G) and adhesion frequency (fraction of curves with adhesion pattern versus all recorded curves) (D/H). **(I)** Adhesion of human monocytes to NETs pre-incubated with antibodies to indicated NET-associated proteins. **(J)** Adhesion of human monocytes to NETs pre-incubated with antibodies to S100A8/9 or isotype-matching IgG. **(K/L)** Human monocytes were incubated with biotinylated histone H2a (10 µg/ml, 15 min, 4°C) and detected using streptavidin APC. Monocyte subsets were identified by CD14, CD16 and Slan staining properties. Quantification of histone $H2a^+$ monocyte subset (K) and representation of histone H2a binding intensity to monocyte subsets (L). Data are analyzed by unpaired t-test (A-D, F, H) or Mann Whitney test (E, G) or one-way ANOVA with

Dunnett's multiple comparisons test (I-L); *p≤0.05, **p≤0.01, ***p≤0.001. All data are presented as mean ±SEM.

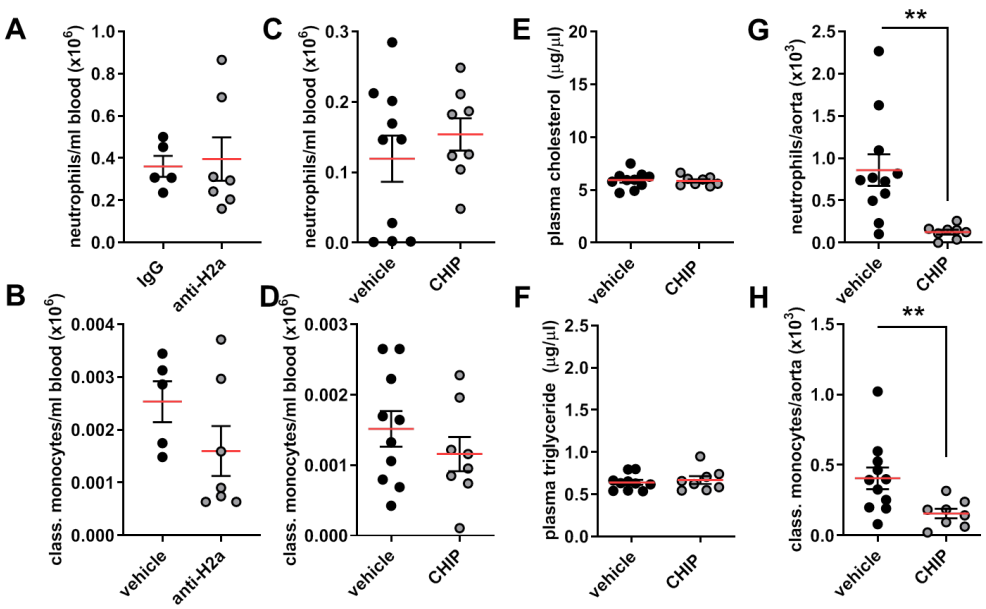

Figure IV: Blocking histone H2a does not affect plasma lipids or cell counts in blood but rather diminishes aortic myeloid cell accumulation. (A-B) *Apoe*$^{-/-}$*Cx*$_3$*cr1*^{GFP} were fed a HFD for 4 weeks, treated with LPS (1 mg/kg, 4h) and injected with isotype respective control (IgG), or a histone H2a-targeting antibody (anti-H2a).Displayed are blood neutrophil (A) and monocyte (B) counts. **(C-H)** *Apoe*$^{-/-}$ *Cx*$_3$*cr1*^{GFP} were fed a HFD for 4 weeks, treated with LPS (1 mg/kg, 4h) and injected with vehicle or CHIP (5 mg/kg). Displayed are blood neutrophil (C) and monocyte (D) counts as well as plasma cholesterol (E) and triglyceride levels (F). Flow cytometry was used to assess neutrophils (G) and classical monocytes (H) in aortic homogenates. Data were analyzed with Mann-Whitney test (A-C) or Unpaired t test (D-H); **p≤0.01. All data are presented as mean ±SEM.