Supplementary Information

Catapult-like release of mitochondrial DNA by eosinophils contributes to anti-bacterial defense

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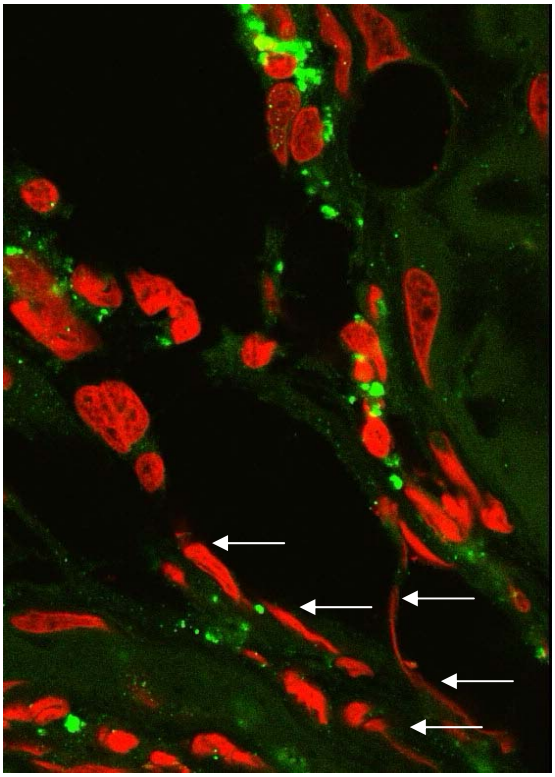
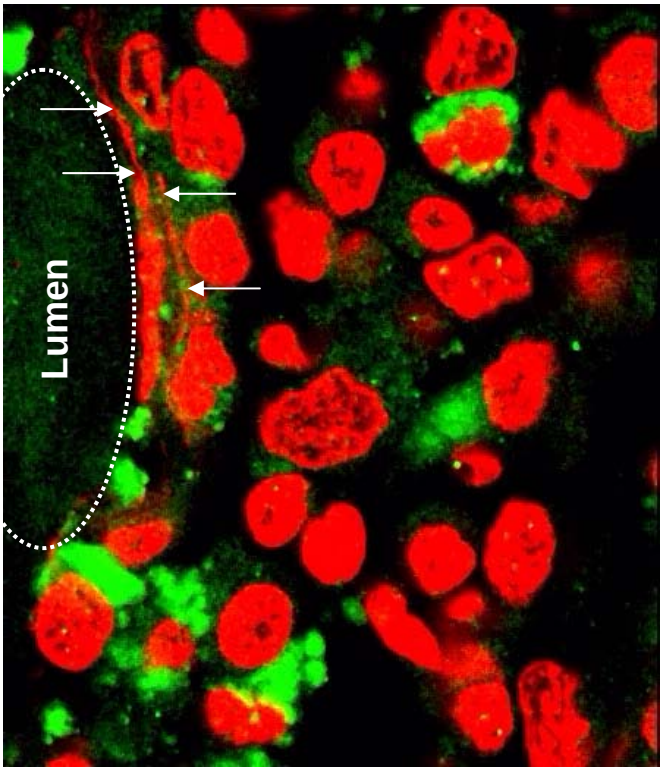
Crohn's disease



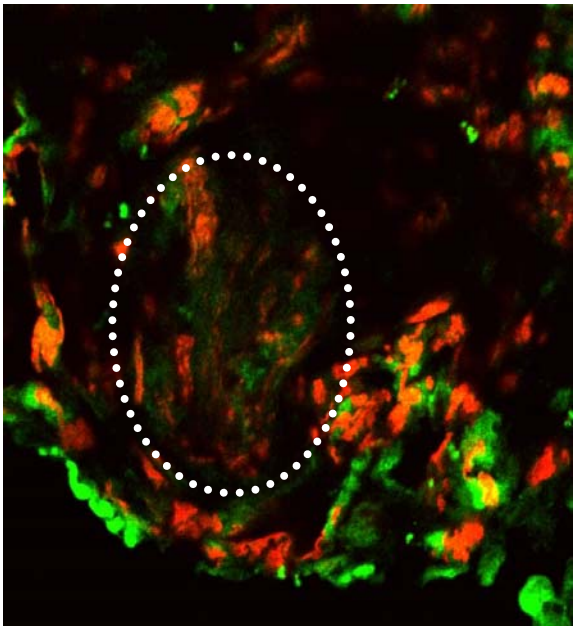
DNA/ECP



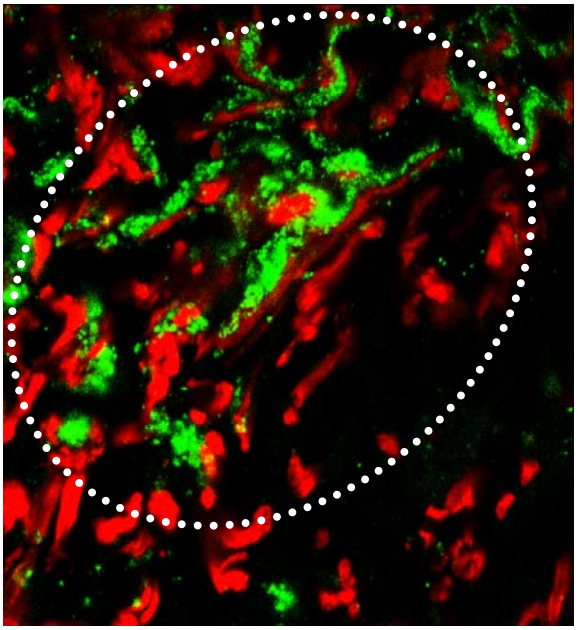
DNA/ECP

Supplementary Movie 1 online.
Demonstration of extracellular DNA and eosinophil granule protein deposition under *in vivo* conditions (Crohn's disease).
DNA was stained with PI and ECP with a specific antibody. With an interval of 0.1 μm, slices were taken throughout the z-axis (z stacks) with 10 (left) and 15 (right) slices per stack in order to show extracellular DNA in their full extension (location is indicated by arrows).

Schistosoma infection



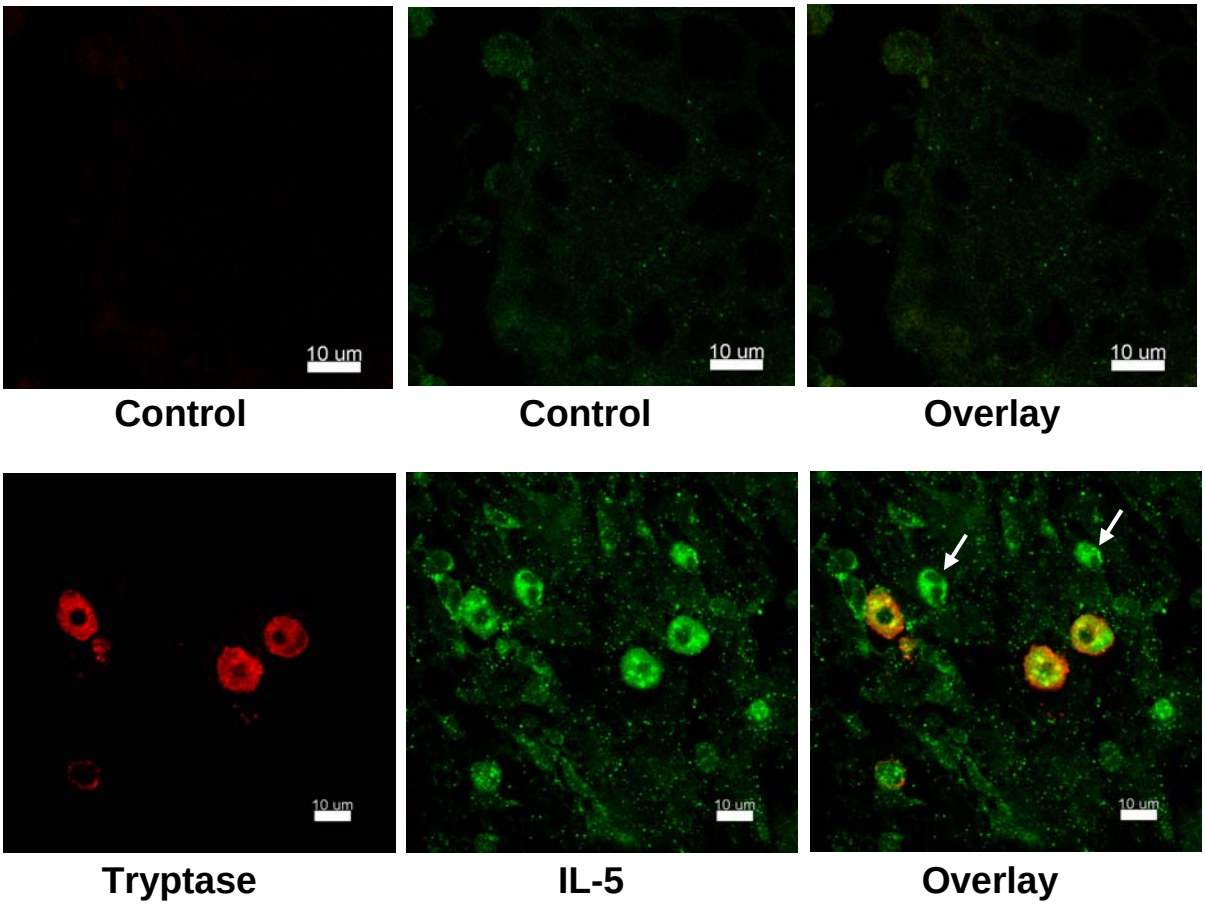
DNA/ECP



DNA/MBP

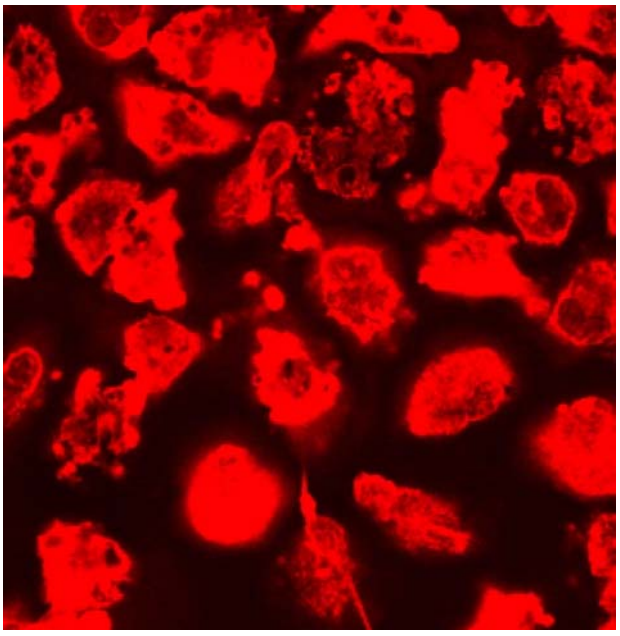
Supplementary Movie 2 online.
Demonstration of extracellular DNA and eosinophil granule protein deposition under *in vivo* conditions (Schistosoma infection).
DNA was stained with PI and ECP/MBP with specific antibodies. With an interval of 0.1 μm, slices were taken throughout the z-axis (z stacks) with 50 (left) and 20 (right) slices per stack in order to show extracellular DNA in their full extension (the location of areas with large extracellular DNA deposition are indicated by dashed circles).

Crohn's disease

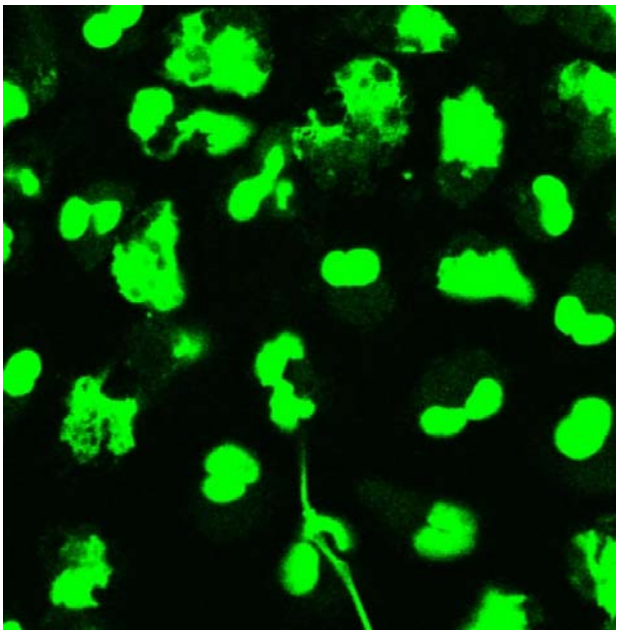


Supplementary Figure 1 online.
Expression of IL-5 in biopsy specimens of patients with Crohn's disease. Mast cells were visualized by a mAb to tryptase. Eosinophils were identified by morphology (arrows). We also observed some smaller IL-5 positive cells in these sections, most likely T cells. The bars represent 10 μm. Results are representative of three independent experiments.

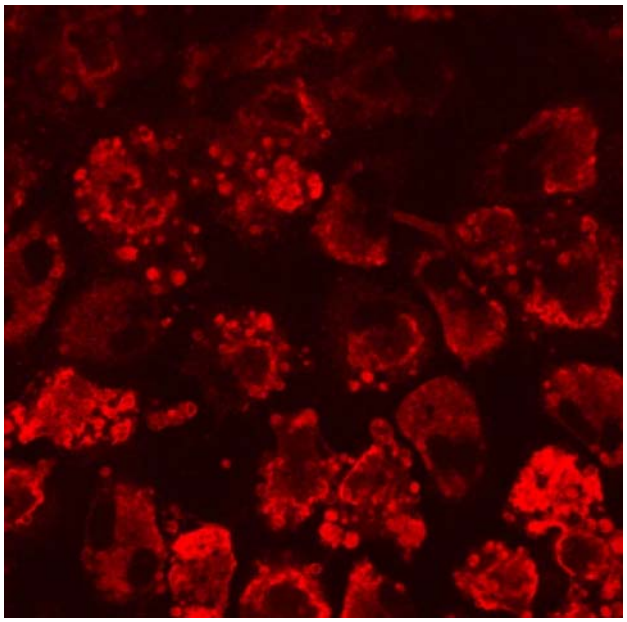
Purified eosinophils



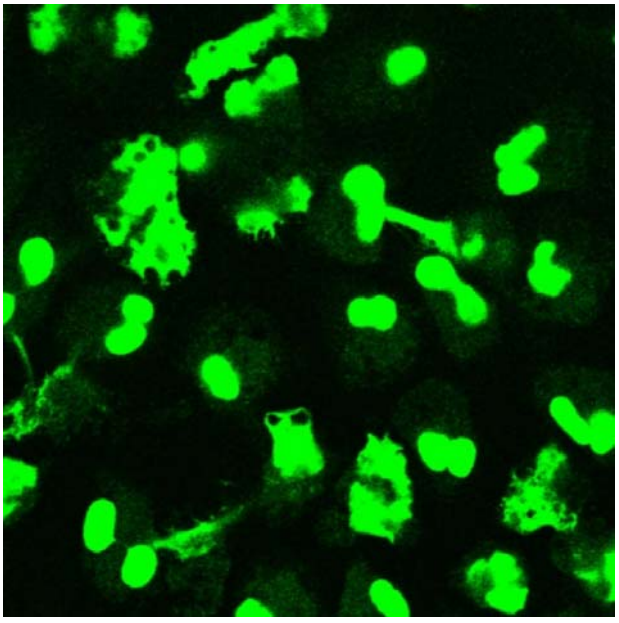
ECP



DNA

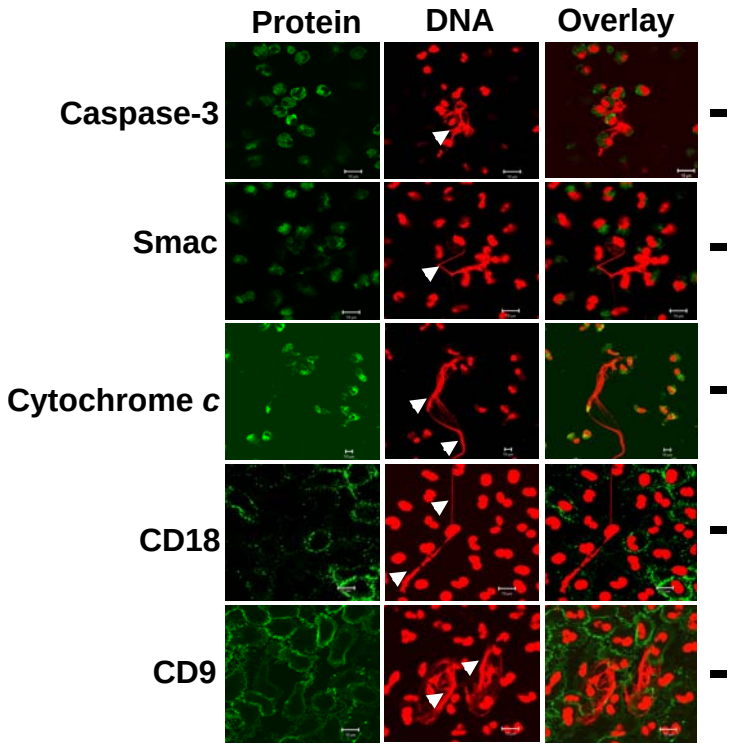


MBP

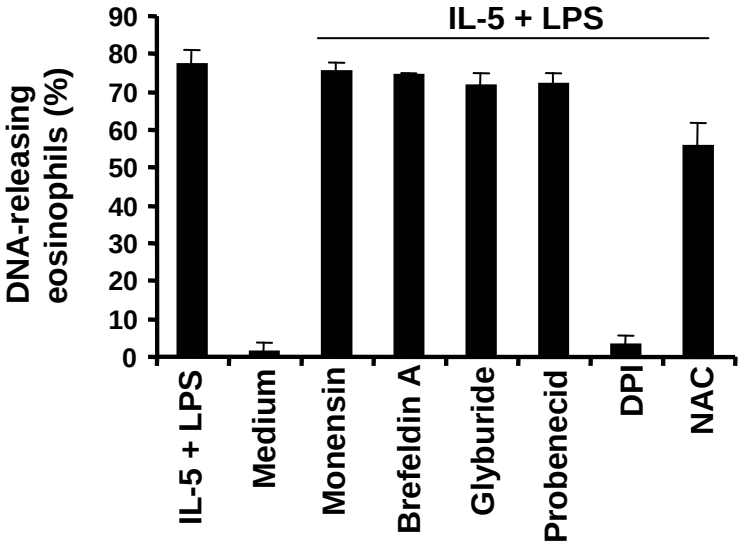


DNA

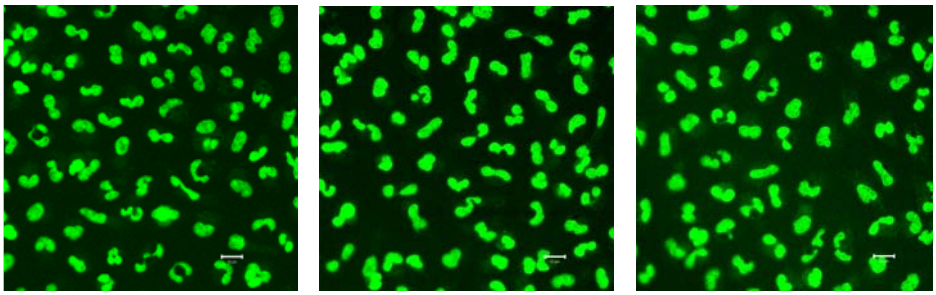
Supplementary Movie 3 online.
Demonstration of extracellular DNA and eosinophil granule protein deposition under *in vitro* conditions.
Highly purified blood eosinophils were stimulated with IL-5/LPS. DNA was stained with PI and granule proteins with specific antibodies. With an interval of 0.1 μm, slices were taken throughout the z-axis (z stacks) with 10 slices per stack in order to show extracellular DNA in their full extension.



Supplementary Figure 2 online.
Eosinophils do not release cytoplasmic, mitochondrial, and membrane proteins. Purified blood eosinophils were activated with IL-5/C5a and stained with PI and the indicated antibodies and subsequently analyzed by confocal microscopy.



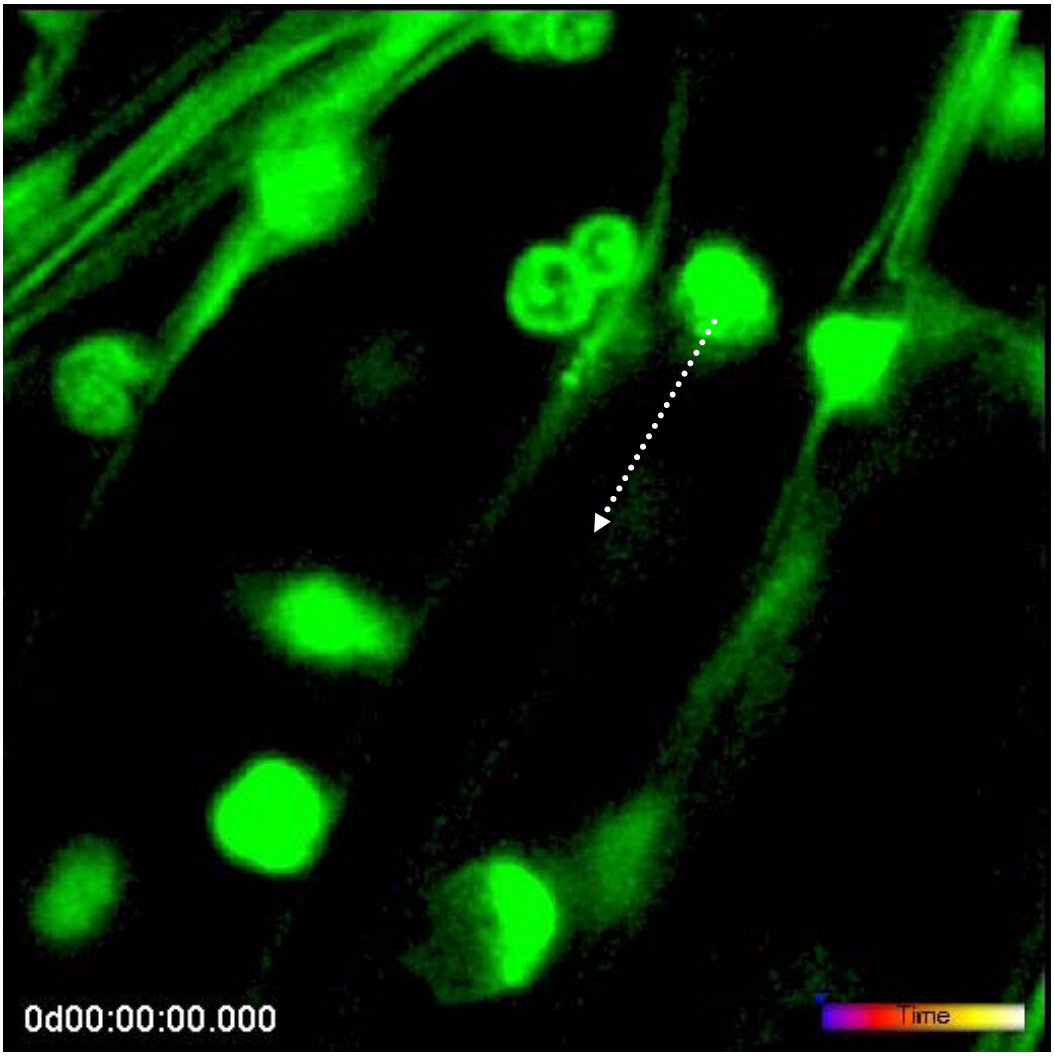
Supplementary Figure 3 online.
Pharmacological inhibition of ROS generation blocks DNA release. IL-5 primed and SYTO 13 stained eosinophils were monitored after LPS stimulation. Cells were analyzed by confocal microscopy in the presence and absence of pharmacological inhibitors. In contrast to DPI, inhibitors of the endoplasmic reticulum/Golgi secretion pathway had no effect (mean ± SEM, n=6).



Medium **IFN-γ + LPS** **IL-5 + LPS**

Supplementary Figure 4 online.
Lack of DNA release by purified blood eosinophils isolated from chronic granulomatous disease (CGD) patients.
Eosinophils were stimulated as indicated. Positive control experiments using normal eosinophils were performed in each case (data not shown). The bars represent 10 μm. DNA was stained by SYTO 13 in all panels. All images are projections of a z stack. Results are representative of three independent experiments.

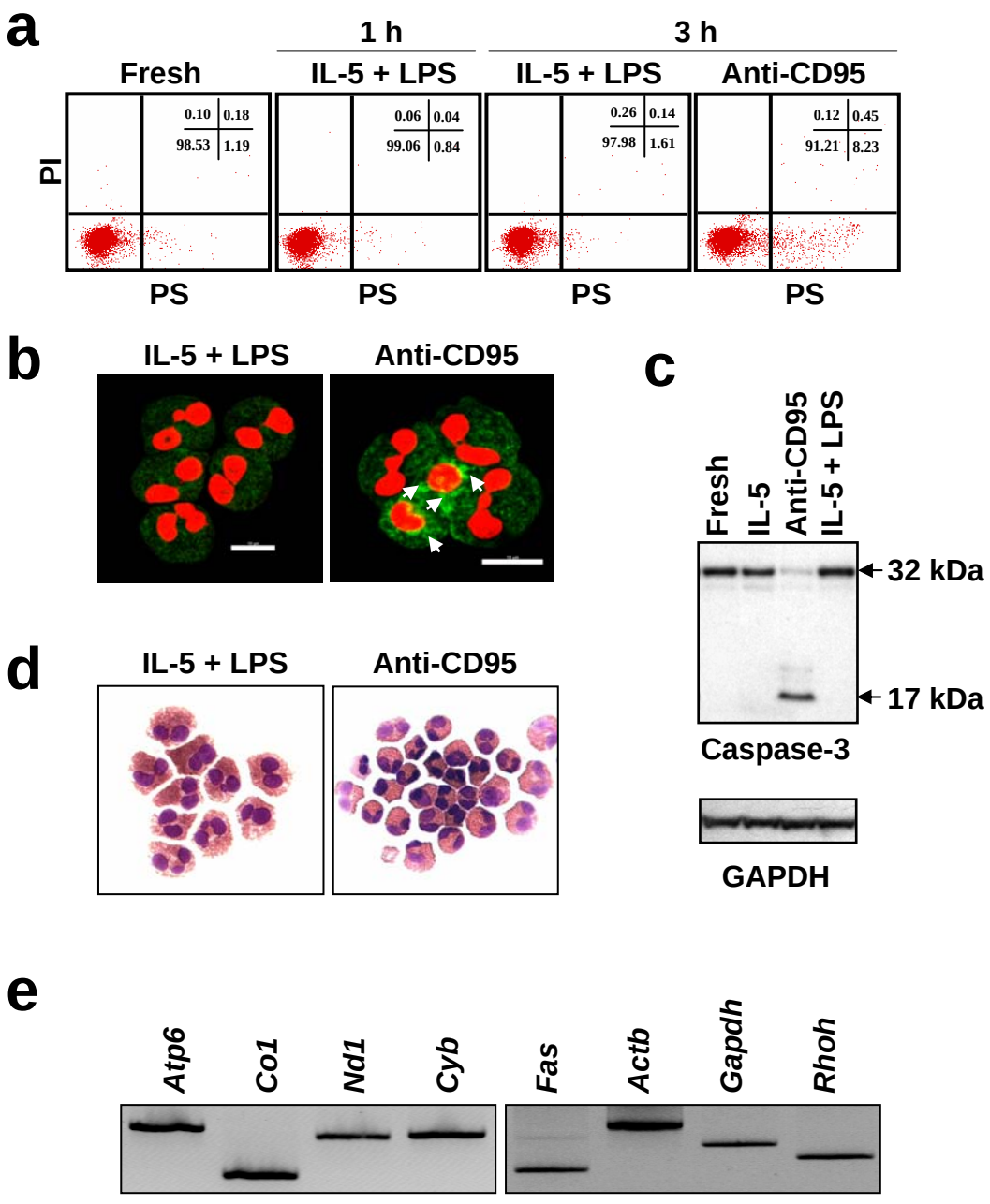
Purified eosinophils



DNA

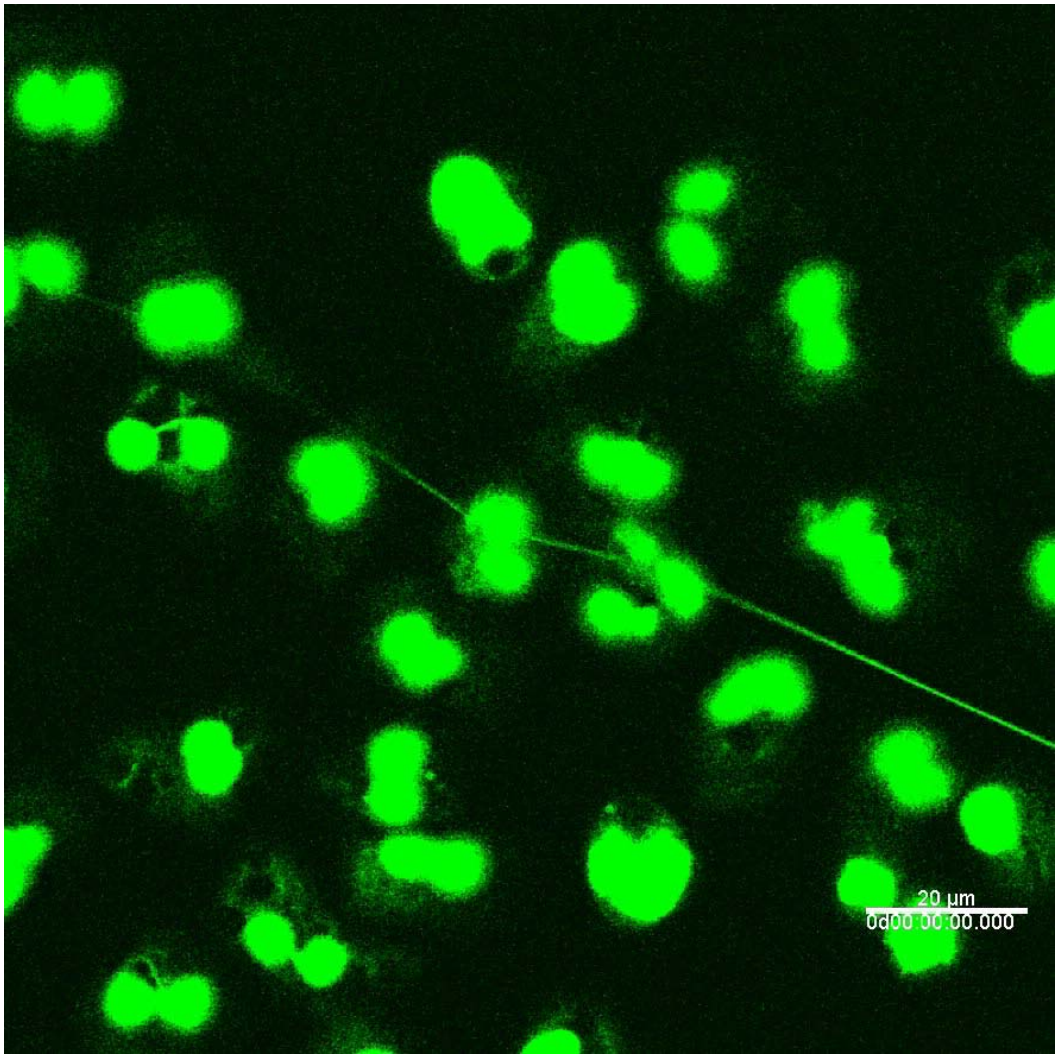
Time-lapse video microscopy

Supplementary Movie 4 online.
Release of DNA from activated eosinophils as assessed by live cell imaging analysis. DNA was stained with SYTO 13. One particular eosinophil (arrow) was followed in a time-dependent manner. Release of DNA occurred with less than 1 second.



Supplementary Figure 5 online.
The release of DNA by IL-5/LPS activated eosinophils is not associated with cell death/apoptosis. (**a**) No detectable phosphatidylserine redistribution up to 3 h stimulation. As a positive control, we used agonistic mAb to CD95 known to induce eosinophil apoptosis. The indicated numbers are relative cell numbers in percentages. (**b**) Negative immunostaining of cleaved caspase-3 in IL-5/LPS - stimulated eosinophils. (**c**) Immunoblotting: No detectable caspase-3 cleavage in IL-5/LPS - stimulated eosinophils. (**d**) No morphological signs of cell death in IL-5/LPS - stimulated eosinophils. Cells in panels (b), (c) and (d) were stimulated for 3 h and anti-CD95 treatment was used for positive controls. The results are representative of at least three independent experiments in each panel. (**e**) Polymerase chain reaction: Eosinophils were cultured for 48 h leading to secondary necrosis. Mitochondrial and nuclear genes were amplified from DNA extracted from cell supernatants (see **Fig. 3**).

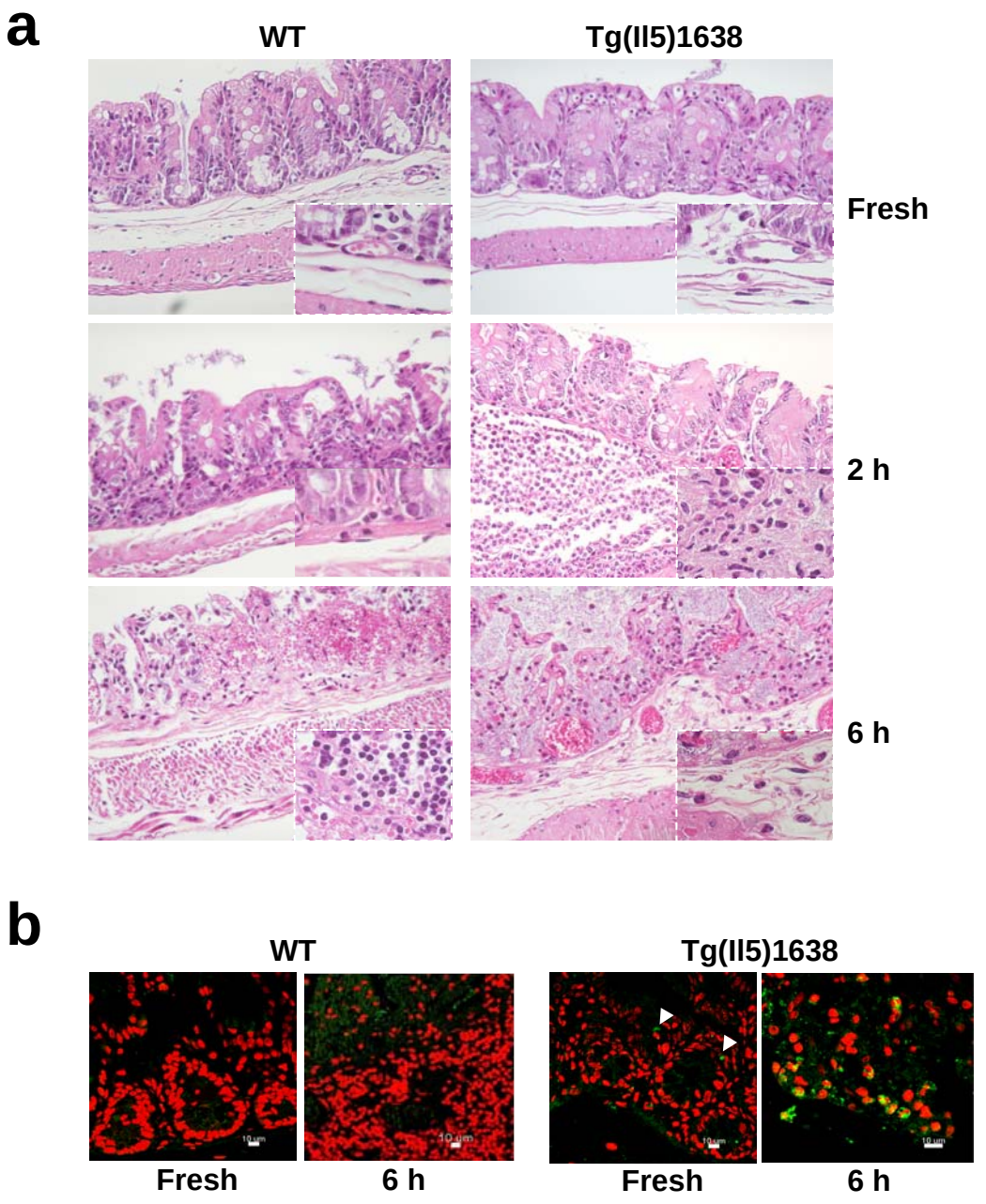
Purified eosinophils



DNA

Time-lapse video microscopy

Supplementary Movie 5 online.
Release of DNA from activated eosinophils as assessed by live cell imaging analysis. DNA was stained with SYTO 13. It appears that the DNA is released from perinuclear but not nuclear structures.


a
WT
Tg(Il5)1638
Fresh
2 h
6 h
b
WT
Tg(Il5)1638
Fresh
6 h
Fresh
6 h
c
Survival (%)
100
80
60
40
20
0
0
24
48
72
96
120
Time (h)
WT + Eos
WT+ Saline
P < 0.05

Supplementary Figure 6 online.
Rapid eosinophil infiltration of the cecum in *IL-5* transgenic mice (Tg(Il5)1638) and anti-bacterial effect of adoptively transferred eosinophils in wild-type (WT) mice after CLP. (**a**) Histologic features of the cecum of Tg(Il5)1638 mice and littermate controls (WT) prior to and at the indicated times after CLP (x250). *Lower right corner:* Higher magnification of infiltrating cells (x1000). Specimens were stained with hematoxylin and eosin. (**b**) Eosinophil infiltration of the cecum prior to and 6 h after CLP. Eosinophils were specifically stained with Ab to MBP (green). Nuclei were counterstained with PI (red). In contrast to WT mice, we detected a few eosinophils (arrow heads) in Tg(Il5)1638 mice prior to CLP. Six h after CLP, strong eosinophil infiltration was observed in Tg(Il5)1638 but not WT mice (see **Fig. 4b,c**). The weakly stained green particles seen in WT mice 6 h after CLP represent red blood cells, demonstrating hemorrhagic inflammation at this time point. The bars represent 10 μm. (**c**) WT mice were administered either saline (n=5) or $2x10^5$ purified eosinophils (n=7) from Tg(Il5)1638 mice *ip* 4 h prior to CLP and monitored for survival. Statistics was performed by Kaplan-Meier analysis.

Supplementary Methods

Reagents. IL-5, IFN-γ, and eotaxin were purchased from R&D Systems. Complement factor 5a (C5a), LPS, propidium iodide (PI) nucleic acid stain, as well as the pharmacological inhibitors monensin sodium, glyburide, brefeldin A, probenecid, N-Acetyl-L-cysteine (NAC), and cytochalasin D were from Sigma-Aldrich. Diphenyleneiodonium (DPI) was from Calbiochem. SYTOX Orange, SYTO 13, MitoTracker Red 580 FM probe, BacLight Green bacterial stain, and 4',6-diamidino-2-phenylidole (DAPI) were obtained from Invitrogen. DNase was from Roche Diagnostics. Monoclonal antibodies (mAbs) to cytochrome *c*, calnexin, and CD9 were purchased from Becton Dickinson (BD) Biosciences. From the same company, we received the Annexin V apoptosis detection kit. MAb to tryptase was from Dako. Polyclonal Abs to pan-caspase-3, active caspase-3, lamin A, and poly(ADP-ribose)polymerase (PARP) were from Cell Signaling. MAb to CD95 (clone CH-11) was obtained from MBL International Corporation. Polyclonal Ab to LPS was from Milan Analytica AG and anti-second mitochondria-derived activator of caspase (Smac) Ab from Imgenex. Polyclonal Ab to IL-5 was from Santa Cruz Biotechnology, Inc. MAb to β-actin was from Sigma-Aldrich and mAb to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Chemicon International. MAb to eosinophil cationic protein (ECP) (EG1) was obtained from Pharmacia Diagnostics and mAb to major basic protein (MBP) (BMK-13) from Cell Sciences, Inc. Affinity-purified rabbit Ab to MBP was generated in our own laboratory[18].

Image and data processing. With an interval of 0.1 μm, slices were taken throughout the z-axis (z stacks) with 10 to 50 slices per stack in order to show the extracellular DNA in its full extension. We deconvoluted the z stacks with Huygens-2 software (Scientific Volume Imaging) using the classic maximum likelihood estimation. Deconvoluted stacks were then used for image analysis by Imaris software to visualize the shapes and sizes of the DNA structures, and their relation to the involved eosinophils. For time-lapse automated confocal imaging, we observed SYTO 13 stained and IL-5 primed eosinophils following LPS or C5a stimulation. For each eosinophil, typical stacks of 512 x 512 pixels x 10 images were acquired for a maximum of 30 min (*xyt* resolution: 0.14 x 0.14 μm x 1 sec). Fluorescence was visualized through a LSM 5 Exciter microscope (AOTF attenuation of 4.8%, laser line 488 nm, PMT voltage 802V, amplifier offset -0.223), using a Plan-Apochromat objective (63x, NA 1.4, oil imersion) and a 120-μm confocal pinhole aperture.

Specific polymerase chain reaction (PCR) conditions. The origin of the extracellular DNA was determined by amplifying four nuclear and four mitochondrial genes. Primers for the following genes were used: *ATP synthase subunit 6 (atp6)* (5'-ATACACAACACTAAAGGACGAACCT-3' and 5'-GAGGCTTACTAGAAGTGTGAAAACG-3'), *cytochrome oxidase c subunit 1 (co1)* (5'-GGAGTCCTAGGCACAGCTCTAA-3' and 5'-GGAGGGTAGACTGTTCAACCTG-3'), *NADH dehydrogenase subunit 1 (nd1)* (5'-GCATTCCTAATGCTTACCGAAC-3' and 5'-AAGGGTGGAGAGGTTAAAGGAG-3'), *cytochrome oxidase b (cyb)* (5'-CTAGCAGCACTCCACCTCCTAT-3' and 5'-GTTGTCCTCCGATTCAGGTTAG-3'), *gapdh* (5'-CCCCTTCATTGACCTCAACTAC-3' and 5'-GAGTCCTTCCACGATACCAAAG-3'), *actb* (5'-ATCTGGCACCACAC CTTCTACAATGAGCTGCG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'), *rhoh* (5'- ATGCTGAGTTCCATCAAGTGCGTGTTG-3' and 5'-TTAGAAGATCTTGCACTC-3'), and *fas* (5'-TCACCACTATTGCTGGAGTCAT-3' and 5'-TAAACATCCTTGGAGGCAGAAT-3'). DNA (100 ng) was amplified with primers at 1 μM. The cycling parameters were as follows: 95^{o}C for 1.5 min, 55^{o}C for 2 min, 72^{o}C for 3 min, 30 cycles. The PCR products were separated on 3% agarose gels and visualized by means of the ethidium bromide staining.