Serum-Derived Plasminogen Is Activated by Apoptotic Cells and Promotes Their Phagocytic Clearance

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Serum-Derived Plasminogen Is Activated by Apoptotic Cells and Promotes Their Phagocytic Clearance

Matthias Rosenwald,*† Uwe Koppe,*† Hildegard Keppeler,* Guido Sauer,‡ Roman Hennel,§ Anne Ernst,§ Karin Erika Blume,* Christoph Peter,*§ Martin Herrmann,§ Claus Belka,§ Klaus Schulze-Osthoff,† Sebastian Wesselborg,*§ and Kirsten Lauber*‡

The elimination of apoptotic cells, called efferocytosis, is fundamentally important for tissue homeostasis and prevents the onset of inflammation and autoimmunity. Serum proteins are known to assist in this complex process. In the current study, we performed a multistep chromatographic fractionation of human serum and identified plasminogen, a protein involved in fibrinolysis, wound healing, and tissue remodeling, as a novel serum-derived factor promoting apoptotic cell removal. Even at levels significantly lower than its serum concentration, purified plasminogen strongly enhanced apoptotic prey cell internalization by macrophages. Plasminogen acted mainly on prey cells, whereas on macrophages no enhancement of the engulfment process was observed. We further demonstrate that the efferocytosis-promoting activity essentially required the proteolytic activation of plasminogen and was completely abrogated by the urokinase plasminogen activator inhibitor-1 and serine protease inhibitor apronin. Thus, our study assigns a new function to plasminogen and plasmin in apoptotic cell clearance. The Journal of Immunology, 2012, 189: 000–000.

Elmination of apoptotic cells by professional phagocytes is a prerequisite for maintaining tissue homeostasis. Rapid engulfment of apoptotic bodies prevents the leakage of intracellular contents that can initiate inflammation and tissue injury (1). Consequently, deranged removal of apoptotic cells is associated with autoimmune diseases, including systemic lupus erythematosus (2). A sophisticated network of “eat-me” signals, phagocytic receptors, and bridging proteins orchestrates apoptotic cell clearance (3). Among the latter, serum proteins, including complement components and protein S, have been reported to play a role (3–5).

Plasminogen, the zymogen of the protease plasmin, is synthesized in the liver and is present at micromolar concentrations in plasma and extracellular fluids (6). It crucially contributes to fibrinolysis and tissue remodeling. For activation, plasminogen is proteolytically converted into plasmin. Activation by tissue-type plasminogen activator leads to lysis of fibrin clots in the blood, whereas urokinase-type plasminogen activator mediates cell surface-associated plasminogen activation (6).

The present study was designed to identify serum proteins promoting apoptotic cell engulfment. By employing a multistep chromatographic fractionation of human serum and mass spectrometry, we identified plasminogen as a novel factor stimulating corpse engulfment.

Materials and Methods

Cells and reagents

Primary human macrophages were generated by differentiation of positively selected (anti-CD14 magnetic beads; Miltenyi Biotec, Bergisch Gladbach, Germany) peripheral monocytes with 20 ng/ml M-CSF or GM-CSF (both from R&D Systems, Heidelberg, Germany, respectively), in macrophage medium (Invitrogen Life Technologies, Heidelberg, Germany) supplemented with 5% autologous serum for 7 d. Human neutrophils were obtained by double-Ficoll gradient purification (Histopaq 1.119 g/ml, Sigma-Aldrich, Deisenhofen, Germany; Ficoll-Paque 1.077 g/ml, GE Healthcare, Freiburg, Germany) and used as prey cells 24 h after preparation, when they had undergone spontaneous apoptosis.

THP-1 cells were differentiated into macrophages with 10 nM PMA (Sigma-Aldrich) for 2.5 d. irradiated with 10 mJ/cm2 UV-C to undergo apoptosis, or heated to 58˚C for 20 min to induce necrosis, respectively (7, 8). Plasminogen, plasmin, thrombin, and plasminogen activator inhibitor–1 (PAI-1) were purchased from American Diagnostica (Heidelberg, Germany), plasminogen FITC was from Biomac (Leipzig, Germany), and plasmin-Cy2 was prepared with the Cy2-labeling kit (GE Healthcare), according to the manufacturer’s instructions. Recombinant human plasmin (produced in yeast) was obtained from Merck Millipore (Darmstadt, Germany). Recombinant human Glu-plasminogen was expressed and purified from an Escherichia coli BL21[DE3] expression clone carrying the cDNA encoding human Glu-plasminogen in pET15b (Merck Calbiochem). The his-tagged protein was purified on a Ni-NTA His-Bind resin column (Merck Calbiochem) and subsequently applied to an EndoTrap blue LPS-removal column (Hyglos, Bernried, Germany). Purity and integrity were confirmed by SDS-PAGE and Coomassie staining. Protein S, kininogen, and all other reagents were obtained from Sigma-Aldrich.

Chromatographic fractionation of human AB serum

All chromatography steps were carried out on a BioLogic FPLC Workstation (Bio-Rad, Munich, Germany) with resins and/or prepacked columns from GE Healthcare. Human AB serum was subjected to native ammonium sulfate precipitation (80% saturation). The resulting protein pellet was resolved in 10 ml 20 mM Tris-HCl (pH 7.4) and 160 mM NaCl and desalted on a HiPrep 26/10 desalting column. The protein fraction of the flow-through was collected at OD280nm >2.0. For removal of IgG, the desalted protein pool (600 mg protein) was passed over a protein G Sepharose column (3.5 ml gel bed). After elution of bound IgG at pH...
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2.6 and re-equilibration of the column, this step was repeated to a total of four times. To reduce the albumin content of the sample, the protein G flow-through (400 mg protein) was passed over a Cibacron Blue affinity column (5 ml Hitrap Blue). The flow-through was collected and combined with the bound nonalbumin proteins eluted at 500 mM NaCl in 20 mM Tris-HCl (pH 7.4). After elution of albumin with 2 M NaCl in 20 mM Tris-HCl (pH 7.4) and re-equilibration of the column, this step was repeated with the flow-through fraction to a total of three runs. The IgG-depleted, albumin-reduced protein pool (90 mg protein in 20 mM bis-Tris-HCl [pH 6.0]) was subjected to cation exchange chromatography on a Mono S HR 5/5 Sepharose column (1 ml gel bed). After washing with 5 column volumes, elution was performed by applying an ascending linear salt gradient (0–800 mM NaCl in 20 mM Tris-HCl [pH 7.4]) over 20 column volumes. Fractions of 500 μl were collected, immediately neutralized to pH 7.4, and analyzed for phagocytosis-promoting activity. The most active peak fractions were pooled and adjusted to 20 mM Tris-HCl (pH 7.4) in 1 M ammonium sulfate for hydrophobic interaction chromatography.

The protein pool obtained in the Mono S separation step was further fractionated on a HiTrap phenyl-Sepharose column (1 ml gel bed). After washing with 5 column volumes, elution was carried out by applying a descending linear salt gradient (1–0 M ammonium sulfate) over 20 column volumes to the final elution buffer (20 mM Tris-HCl [pH 7.4], 10% glycerol). Fractions of 500 μl were collected and analyzed for their phagocytosis-promoting activity. The most active fractions were pooled on the basis of the OD_{280nm} profile and concentrated on MultiScreen Ultraloc-10 Filter Plates (MWCO 10 kDa; Millipore) against serum-free culture medium.

Mass spectrometry

After SDS-PAGE and silver-staining, bands of interest were excised, destained, and in-gel digested with trypsin (Promega, Heidelberg, Germany) following a published protocol (9). Extracted peptides were desalted with self-made microcolumns (10) and separated by reversed-phase HPLC (nanoLC2D; Eksigent, Dublin, CA) using a capillary column with 15 cm length and 75 μm i.d. SilicaTip (PicoTip; New Objective, Woburn, MA) self-packed with ReproSil-Pur C18-AQ, 3 μm (Maisch, Ammerbuch-Entringen, Germany). Mass spectrometric analysis was performed on an ion trap (HDX trap; Bruker Daltonics, Bremen, Germany) equipped with a nanoESI source from Proxeon Biosystems (Odense, Denmark).

Mascot generic data files were created using DataAnalysis (Bruker) and searched against the SwissProt protein database (V54.2) using Mascot Server (V2.2). The following settings were used: digestion with trypsin allowing one miss cleavage, carbamylation of cysteine as fixed modification, oxidation of methionine as variable, 0.3-Da peptide, and fragmentation mass accuracy.

Flow cytometric measurement of phosphatidylserine exposure, plasminogen, and plasmin binding

Phosphatidylserine (PS) exposure was flow cytometrically measured by annexin V FITC/propidium iodide (PI) staining (annexin V-FLUOS staining kit; Roche, Penzberg, Germany). Cells with positive annexin V FITC, but negative PI signal were considered apoptotic, whereas cells double positive for annexin V FITC and PI staining were considered necrotic (7).

To analyze surface binding, plasminogen FITC or plasmin Cy2 (10 μg/ml) were incubated with 1 × 10^6 cells in serum-free medium for 20 min at room temperature. Afterward, cells were collected by centrifugation, resuspended in serum-free medium supplemented with 2 μg/ml PI, and subjected to FACS analysis.

Phagocytosis assay

Phagocytosis was measured, as described previously (7, 11). Briefly, 1 × 10^7 prey cells/ml were labeled with 2 μM PKH26 in diluent C (Sigma-Aldrich) for 5 min at room temperature, according to the manufacturer’s instructions. After extensive washing, prey cells were irradiated with 10 mJ/cm² UV-C and incubated for 12 h (if not stated otherwise) in serum-free medium, or left untreated for viable controls. Subsequently, prey cells were added to 1 × 10^5 PKH67-stained, PMA-differentiated THP-1 macrophages or primary monocyte-derived macrophages per well in 24-well plates in a 2:1 ratio (if not stated otherwise) and incubated for 2 h in serum-free medium. Finally, cells were harvested by trypsinization and analyzed by flow cytometry. Phagocytosis was measured as the percentage of macrophages with internalized prey cells (double positive for PKH67 and PKH26 staining) on the basis of all macrophages deployed (positive for PKH67 staining).

SDS-PAGE, Coomassie staining, silver staining, and immunoblot analyses

SDS-PAGE, Coomassie, silver staining, and immunoblot analyses were performed, as described previously (12). Anti-human poly(ADP-ribose) polymerase Ab was obtained from Enzo Life Sciences (Lörrach, Germany), and anti-human caspase-3 Ab was from BD Biosciences (Heidelberg, Germany).

Plasmin activity test

A total of 5 × 10^5 cells was incubated with plasminogen in serum-free medium at 37°C for 2 h. Cell-free supernatants were collected and assessed for plasmin activity with 100 μM fluorogenic peptide substrate ALK-amc (Fremont, CA). The increase in fluorescence was monitored at 37°C over 1 h. Plasmin activity was calculated from the baseline-corrected slope of the curves and is given as FU/min.

Depletion of plasminogen from human AB serum

Plasminogen was specifically depleted from human AB serum via lysis-Sepharose chromatography, according to the manufacturer’s instruction (GE Healthcare). Removal of plasminogen was monitored by SDS-PAGE with subsequent Coomassie staining and quantitative analysis on a Licor Odyssey Fluorescence Imaging System.

FIGURE 1. Serum-derived factors promote apoptotic cell engulfment. (A) Human AB serum enhances the phagocytosis of apoptotic cells by macrophages in a dose-dependent manner. Apoptotic, PKH26-labeled THP-1 monocytes were incubated with PMA-differentiated, PKH67-labeled THP-1 macrophages in a 2:1 ratio for 2 h in the presence of 0–10% (v/v) serum, before phagocytosis of prey cells was assessed by flow cytometry. (B) Engulfment-enhancing effect of serum at different macrophage/prey ratios. **p < 0.01, unpaired two-tailed Student t test analysis of serum-free versus serum-containing samples at the respective prey/macrophage ratio. (C) Serum-derived phagocytosis-enhancing factors act mainly on the prey cells. Macrophages or prey cells were separately preincubated with 5% (v/v) serum for 30 min. Cells were washed and phagocytosis was analyzed in parallel in samples with and without serum addition. **p < 0.01, unpaired two-tailed Student t test. (D) Complement and noncomplement factors contribute to the serum-derived phagocytosis-enhancing effect. Human AB serum was left untreated (native) or was decomplemented by heat treatment (56°C, 30 min). Subsequently, the serum samples were added at a final concentration of 5% (v/v) for a phagocytosis assay. **p < 0.01, unpaired two-tailed Student t test.
Data analyses and statistics

If not stated otherwise, results are displayed as mean values ± SD of intra-assay replicates of one representative of at least three independent experiments. The p values were calculated by heteroskedastic, unpaired, two-tailed Student t test, and asterisks indicate \( p < 0.01 \) (***) or \( p < 0.05 \) (*), respectively.

Results

Addition of human serum augments the engulfment of apoptotic cells

Several serum proteins, including complement proteins and protein S, have been described to play a role in apoptotic cell clearance (3–5, 13). We examined in depth the influence of human serum on apoptotic cell engulfment as measured by a flow cytometric phagocytosis assay (Supplemental Fig. 1A–D) and observed that serum enhanced the phagocytic uptake of apoptotic cells in a dose-dependent manner (Fig. 1A, Supplemental Fig. 2A). This effect was clearly detected at different macrophage/prey cell ratios in phagocytosis assays employing PMA-differentiated THP-1 macrophages engulfing apoptotic THP-1 monocytes or primary human macrophages engulfing autologous apoptotic neutrophils, respectively (Fig. 1B, Supplemental Fig. 2B). Moreover, serum pre-incubation of either macrophages or prey cells revealed that the serum effect on phagocytosis targets prey cells rather than macrophages (Fig. 1C). Complement inactivation (heat treatment with 56°C for 20 min) partially attenuated the phagocytosis-enhancing effect, but heat-treated serum still significantly promoted apoptotic cell engulfment (Fig. 1D).

Chromatographic fractionation of the phagocytosis-promoting factors in human AB serum

To identify the responsible serum factor(s), we performed a multi-step chromatographic separation of human serum (Fig. 2A). The protein extract obtained by ammonium sulfate precipitation was IgG depleted and reduced in albumin by affinity chromatography. The resulting protein fraction was subjected to a two-step separation

![Flow chart illustrating the chromatographic separation strategy](http://www.jimmunol.org/)

**FIGURE 2.** Identification of plasminogen as a serum-derived factor promoting apoptotic cell engulfment. (A) Flow chart illustrating the fractionation strategy. (B) Mono S cation exchange chromatogram. Fractionation of the IgG-depleted, albumin-reduced protein fraction of human AB serum (90 mg protein) was performed. Elution was monitored on the basis of OD280nm and conductivity. Fractions with highest phagocytosis-enhancing activity (gray area) were combined and further purified by hydrophobic interaction chromatography. (C) Phenyl-Sepharose chromatogram. The active fraction obtained after Mono S chromatography (500 μg protein) was loaded on a phenyl-Sepharose column. Elution was monitored on the basis of OD280nm and conductivity. Fractions with highest phagocytosis-enhancing activity (gray area) were pooled and concentrated by ultrafiltration. The gray-shaded protein fraction was analyzed by SDS-PAGE and subsequent mass spectrometry. (D) SDS-PAGE of the serum fraction enriched for prophagocytic activity following cation exchange and hydrophobic interaction chromatography. After SDS-PAGE, proteins were visualized by silver staining and identified by mass spectrometry. Most abundant proteins are depicted. Plasminogen was identified with sequence coverage of 47% to be one of the major components.
using cation exchange and hydrophobic interaction chromatography (Fig. 2B, 2C). Fractions with the highest phagocytic activity from each purification step were collected. Subsequent SDS-PAGE revealed four major protein bands that were analyzed by mass spectrometry. Apart from albumin and fragments of the complement proteins C3 and C4, plasminogen was unambiguously identified (Fig. 2D). It should be noted that protein S, a serum protein with known efferocytosis-promoting activity, was found in a different fraction pool than plasminogen (Fig. 2D).

**Plasminogen crucially contributes to the phagocytosis-promoting effect of serum**

To characterize the role of plasminogen in apoptotic cell removal, we used purified plasminogen and observed that it dose dependently stimulated the uptake of apoptotic, but not viable cells by PMA-differentiated THP-1 macrophages as well as M-CSF– or GM-CSF–differentiated primary human macrophages (Fig. 3A, Supplemental Fig. 2C). Similar to protein S, plasminogen promoted phagocytosis even at doses significantly lower than its serum concentration (Fig. 3A). Notably, plasminogen did not alter PS exposure on apoptotic cells (data not shown). The purity of commercially purchased plasminogen was confirmed by SDS-PAGE, Coomassie, and silver staining (Supplemental Fig. 3A, 3B). However, to exclude that nondetectable contaminants in purified plasma-derived plasminogen contributed to the measured enhancement of apoptotic cell engulfment, we also tested purified recombinant human Glu-plasminogen and observed a comparable increase in phagocytosis as in the case of plasma-derived plasminogen (Supplemental Fig. 3C).

Intriguingly, plasminogen-depleted serum stimulated apoptotic cell uptake significantly less efficiently than the same serum reconstituted with plasminogen or native serum, respectively (Fig. 3B, 3C). Similar to complete serum (Fig. 1C), plasminogen-dependent engulfment enhancement was exerted primarily on prey cells, but not on macrophages (Fig. 4A). However, we did not detect enhanced binding of FITC-labeled plasminogen to apoptotic cells in comparison with viable cells, whereas necrotic cells strongly bound plasminogen (Fig. 4B). Hence, the plasminogen-mediated effect on
Efferocytosis was not due to opsonization or coating. Apart from opsonization, plasminogen might support corpse engulfment by its zymogen protease activity. To address this issue, viable and apoptotic cells were incubated with plasminogen, and subsequently, plasmin activity was measured. Intriguingly, although apoptotic and viable cells did not differ in plasminogen binding, a profound activation of plasminogen by apoptotic cells was observed (Fig. 4C). We next examined whether the proteolytic activity of plasmin is required for its phagocytosis-enhancing effect. Phagocytosis of apoptotic THP-1 monocytes by PMA-differentiated THP-1 macrophages was assessed in the absence or presence of plasminogen at 0–10% of its physiological serum concentration (100% serum concentration correlates to 120 μg/ml) with or without addition of 10 μg/ml serine protease inhibitor aprotinin (left panel) or 2 μg/ml PAI-1 (right panel). *p < 0.05 and **p < 0.01 (respectively) in unpaired two-tailed Student t test analysis of inhibitor-containing versus plasminogen-only samples at the corresponding plasminogen concentrations.

**FIGURE 4.** Activation of plasminogen by apoptotic cells is required for its phagocytosis-promoting effect. (A) Plasminogen exerts its phagocytosis-enhancing effect mainly on the prey cells. PMA-differentiated THP-1 macrophages or THP-1 prey cells alone were preincubated in the presence or absence of 12 μg/ml plasminogen. Phagocytosis was then analyzed in parallel in samples with or without plasminogen. Differences in phagocytosis in comparison with the control without plasminogen are shown. **p < 0.01, unpaired two-tailed Student t test. (B) Apoptotic cells do not display an enhanced binding of plasminogen. Viable, apoptotic, and necrotic THP-1 cells were incubated with 10 μg/ml plasminogen FITC, and binding was monitored by FACS analysis. PI exclusion staining was used to monitor plasma membrane integrity. (C) Incubation with apoptotic, but not viable cells leads to activation of plasminogen. Viable and apoptotic THP-1 cells or a serum-free medium control were incubated with different concentrations of plasminogen (100% serum concentration correlates to 120 μg/ml) at 37˚C for 2 h. Then cell-free supernatants were collected and analyzed for plasmin activity with ALK-amc peptide. Mean values of duplicates are shown. (D) Plasminogen activation is required for the phagocytosis-enhancing effect. Phagocytosis of apoptotic THP-1 monocytes by PMA-differentiated THP-1 macrophages was assessed in the absence or presence of plasminogen at 0–10% of its physiological serum concentration (100% serum concentration correlates to 120 μg/ml) with or without addition of 10 μg/ml serine protease inhibitor aprotinin (left panel) or 2 μg/ml PAI-1 (right panel). *p < 0.05 and **p < 0.01 (respectively) in unpaired two-tailed Student t test analysis of inhibitor-containing versus plasminogen-only samples at the corresponding plasminogen concentrations.

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Finally, we addressed the question, in which phase of apoptosis plasmin(ogen) exerts efferocytosis-promoting effect. To this end, we employed prey cells in different stages of the apoptotic progress as measured by PS exposure, caspase, and caspase-substrate processing (Supplemental Fig. 1E, 1F). Notably, these cells had not transited into secondary necrosis, as was confirmed by PI exclusion staining. When applied in a phagocytosis assay, we detected no plasminogen-mediated efferocytosis enhancement in case of the early apoptotic cells 4 h after apoptosis induction (Fig. 6). The onset of plasminogen-mediated phagocytosis promotion was observed with prey cells 8 h after apoptosis induction (when PS exposure had reached its saturation level), passed through a maximum with prey cells 12 h after apoptosis induction, and declined again with prey cells 16 h after apoptosis induction. These data suggest that plasmin (ogen) can only support the engulfment of apoptosing cells, which readily expose “eat-me” signals, such as externalized PS.
Discussion

By employing a multistep chromatographic fractionation of human serum, we have identified plasminogen as a novel factor that promotes the phagocytic removal of apoptotic cells. Serum concentrations of plasminogen are $\sim 120 \, \mu g/ml$, which is $\sim 10$-fold higher than the concentration required to stimulate efferocytosis. Furthermore, we have shown that in this context the proteolytic activation/activity of plasmin(ogen) is of crucial importance. The question that arises is how plasminogen exerts its effect on corpse clearance. Previous reports have described an enhanced binding of plasminogen to the surface of apoptotic cells, suggesting that it might function as an opsonin (14–17). In the current study, however, we did not observe enhanced surface binding of plasminogen to apoptotic cells. This might be explained by the conditions or cell types used. For instance, in previous reports, enhanced plasminogen binding paralleled the loss of plasma membrane integrity as a late event of apoptosis, whereas our analyses included only early, apoptotic cells with an intact plasma membrane. Instead of acting as an opsonin, we show that plasminogen enhances efferocytosis under the crucial contribution of its proteolytic activity, which is acquired after interaction with apoptotic cells. Our data are in line with studies from other groups that have attributed this activation of plasminogen to an increased expression of urokinase-type plasminogen activator, which was specifically detected on the surface of apoptotic, but not necrotic cells (15–17). Notably, plasmin(ogen)-mediated enhancement of dying cell engulfment was observed in a phase of apoptosis, in which the maximum level of PS externalization had already been reached and the integrity of the plasma membrane was still intact, suggesting that the exposure of “eat-me” signals is a prerequisite for plasmin(ogen)-dependent phagocytosis promotion.

The mechanisms responsible for plasmin(ogen)’s prophagocytic effect remain elusive. Nevertheless, plasmin’s cleavage specificity is limited to lysine and arginine residues, and interestingly, gingipain R and clostripain, bacterial proteases with cleavage specificity after arginine, have been described to enhance apoptotic cell phagocytosis as well (18). Therefore, similar substrates might be cleaved by these proteases. One such putative target was proposed to be the “don’t-eat-me” signal CD31 (19), because CD31 surface staining declined after gingipain and clostripain treatment (18). Whether a “don’t-eat-me” signal is indeed degraded or a novel “eat-me” signal is generated by the bacterial proteases and plasmin(ogen) remains to be investigated.

Overall, we have identified a novel and hitherto undescribed activity for plasmin(ogen) in apoptotic cell clearance. Notably, the urokinase plasminogen activator receptor has recently been described as a novel engulfment receptor (20), although its prophagocytic role remains controversial (21). Moreover, Das and Plow (22) have described the histone 2B-dependent recruitment of plasminogen.
to PS-rich surfaces, and it has been shown that PAI impairs apoptotic cell removal (23). These studies taken together with our work sug-
ggest that dysregulation of the plasminogen system might lead to
deranged apoptotic cell clearance. Of note, plasminogen-deficient
mice display delayed involution of the mammary glands (24, 25).
This process is closely linked to apoptotic cell removal and com-
parably impaired in mice lacking the engulfment factor MFG-E8
(26). Further studies will help to elucidate whether plasminogen
deficiency is associated with defects in corpse clearance and the
onset of chronic inflammation and autoimmunity.

Disclosures
The authors have no financial conflicts of interest.

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The authors have no financial conflicts of interest.

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Running title: Plasminogen promotes apoptotic cell clearance
Supplemental Figure 1: Establishment of a flow cytometric assay for the detection of apoptotic cell phagocytosis. (A) The phagocytosis assay detects active, actin-dependent prey cell internalization. PKH67-labeled, PMA-differentiated THP-1 macrophages (100,000 cells/well in a 24-well plate) were co-incubated with 200,000 PKH26-labeled, apoptotic THP-1 cells (12 h post 10 mJ/cm² UV-C) in the absence or presence of 100 μM cytochalasin D (Cyto D) at 37°C for 2 h. Cells were collected by trypsinization and analyzed by flow cytometry. Phagocytosis was calculated as percentage of PKH67/PKH26-double-positive relative to the total number of PKH67-positive macrophages (analysis gate is shown in grey). Representative dotplots of quadruplicates are shown. (B) Time and temperature dependency of the phagocytosis process. Apoptotic THP-1 monocytes and THP-1 macrophages were co-incubated at a ratio of 2:1 at the indicated temperatures for 0 - 120 min. Cells were then harvested and phagocytosis was assessed as in (A). (C) Apoptotic cells are more efficiently engulfed than viable cells. Phagocytosis assay was carried out as described in (A) with viable or apoptotic (12 h post 10 mJ/cm² UV-C) prey cells. (D) Measurement of PS externalization. THP-1 cells were left untreated or induced to undergo apoptosis by UV irradiation (10 mJ/cm²) as described in (C). After 12 h PS externalization was measured by double staining with annexin V-FITC and propidium iodide (PI). The percentage of annexin V-positive, PI-negative cells is displayed. (E) Kinetics of PS externalization after UV irradiation (10 mJ/cm²) of THP-1 cells as measured by double staining with annexin V-FITC and PI. (F) Kinetics of caspase-3- and PARP-processing during UV-induced apoptosis. THP-1 cells were UV-irradiated as in (D). Whole cell lysates were prepared, and 150 μg protein were subjected to 6-15% SDS-PAGE with subsequent immunoblot analysis against caspase-3 and the caspase substrate PARP. Filled arrows indicate the full length proteins and open arrows indicate the cleavage products.
Supplemental Figure 2: Serum and serum-derived plasminogen promote the phagocytosis of apoptotic autologous neutrophils by human monocyte-derived macrophages. (A) Dose-dependency of AB serum-mediated phagocytosis promotion. Apoptotic, PKH26-labeled human neutrophils were incubated with M-CSF-differentiated, PKH67-labeled primary human macrophages in a 2:1 ratio for 2 h in the presence of 0 - 10% (v/v) serum, before phagocytosis of neutrophils was assessed by flow cytometry. (B) Engulfment-enhancing effect of serum at different macrophage:prey ratios. Phagocytosis of apoptotic neutrophils by primary human M-CSF-differentiated macrophages was measured in the presence or absence of 5% (v/v) serum employing different macrophage:neutrophil ratios (4:1 to 1:4). Asterisks depict p<0.01 (unpaired two-tailed Student's t-test analysis of serum-free vs. serum-containing samples at the respective prey : macrophage ratios). (C) Phagocytosis-enhancing effect of purified plasminogen. Phagocytosis of autologous, apoptotic neutrophils by primary human M-CSF or GM-CSF differentiated macrophages was assessed in the absence or presence of plasminogen as described in Fig. 3A. Asterisks indicate p<0.05 (unpaired two-tailed Student's t-test analysis of plasminogen-containing samples vs. the serum-free control).
Supplemental Figure 3: Addition of purified plasma-derived or recombinant plasminogen and plasmin promotes efferocytosis. (A) Purity and integrity of commercially purchased proteins (1 µg per lane) were checked by non-reducing SDS-PAGE and subsequent Coomassie staining. Plasminogen (Plg, 90 kDa), plasmin (Plm, 83 kDa), thrombin (Thr, 37 kDa), protein S (69 kDa). (B) Purity of plasma-derived plasminogen was further confirmed by silver staining. (C) Phagocytosis-enhancing effect of plasma-derived and recombinant human plasminogen and plasmin. Phagocytosis of apoptotic and viable THP-1 monocytes by PMA-differentiated THP-1 macrophages was assessed in the absence or presence of the indicated serum concentrations of purified plasma-derived plasminogen, recombinant human Glu-plasminogen (purified from E. coli), or recombinant human plasmin (purified from yeast). 100% serum concentration correlates to 120 µg/ml plasminogen. Asterisks depict p<0.01 (**) or p<0.05 (*), respectively (unpaired two-tailed Student's t-test analysis of plasminogen-containing samples vs. the serum-free control).