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Cell Surface Externalization of Annexin A1 as a Failsafe Mechanism Preventing Inflammatory Responses during Secondary Necrosis

Karin E. Blume,* Szabolcs Soeroes,* Michaela Waibel,* Hildegard Keppeler,* Sebastian Wesselborg,* Martin Herrmann,† Klaus Schulze-Osthoff,2‡ and Kirsten Lauber2,3*

The engulfment of apoptotic cells is of crucial importance for tissue homeostasis in multicellular organisms. A failure of this process results in secondary necrosis triggering proinflammatory cytokine production and autoimmune disease. In the present study, we investigated the role of annexin A1, an intracellular protein that has been implicated in the efficient removal of apoptotic cells. Consistent with its function as bridging protein in the phagocyte synapse, opsonization of apoptotic cells with purified annexin A1 strongly enhanced their phagocytic uptake. A detailed analysis, however, surprisingly revealed that annexin A1 was hardly exposed to the cell surface of primary apoptotic cells, but was strongly externalized only on secondary necrotic cells. Interestingly, while the exposure of annexin A1 failed to promote the uptake of these late secondary necrotic cells, it efficiently prevented induction of cytokine production in macrophages during engulfment of secondary necrotic cells. Our results therefore suggest that annexin A1 exposure during secondary necrosis provides an important failsafe mechanism counteracting inflammatory responses, even when the timely clearance of apoptotic cells has failed. The Journal of Immunology, 2009, 183: 8138–8147.

Billions of cells die by apoptosis during daily tissue regeneration. Under physiological conditions, apoptotic cells are swiftly recognized, internalized, and degraded by neighboring cells and professional phagocytes (1). However, if the removal process fails, apoptotic cells undergo postapoptotic, secondary necrosis and release potentially cytotoxic and antigenic intracellular contents that can promote inflammation and autoimmunity. Consequently, diverse autoimmune diseases, such as systemic lupus erythematosus, and chronic inflammatory conditions, such as rheumatoid arthritis, have been linked to a deficient and delayed clearance of apoptotic cells (2, 3). Secondary necrosis may not only occur due to intrinsic defects in phagocytic function, but also when massive apoptosis overwhelms the available scavenging capacity. Such situations have been described in acute inflammation associated with massive neutrophil accumulation, ischemia, drug-induced hepatitis, or bacterial infections (3).

To guarantee the efficient removal of apoptotic cells, a complex network of interactions between the apoptotic cell and the phagocyte, the phagocytic synapse, has evolved. Secreted “find-me” signals, exposed “eat-me” signals, bridging proteins and the corresponding phagocyte receptors constitute a plethora of signaling molecules involved in this process. A central event comprises the exposure of phosphatidylserine (PS)4 on the surface of the apoptotic cell (4). PS can directly be bound by various phagocyte receptors, including BAII, TIM1/4, or stabilin-2 (5–8). Alternatively, so-called bridging proteins can opsonize apoptotic cells for their uptake by phagocytes.

One of these bridging proteins is annexin A1 (anx A1), an intracellular protein, which is externalized during cell death and can be found on PS-rich plaques on the surface of dying cells (9, 10). Anx A1 shares the common organizational theme of all annexins involving a highly conserved annexin core domain and a variable N-terminal region (11). The annexin core comprises four annexin repeats, which form a curved protein structure and contain the calcium/phospholipid-binding sites. The 46-amino acid long, unique N-terminus of anx A1 inserts into the protein core and upon calcium binding is pushed out by a conformational change. Apart from its function as a bridging protein for apoptotic cell removal, anx A1 participates in the regulation of the immune system and has been attributed an anti-inflammatory role at various control levels (12).

Deficiencies in apoptotic cell removal and the subsequent accumulation of secondary necrotic debris have been reported to promote the development of chronic inflammation and autoimmunity (2, 3). Consequently, strategies improving dying cell removal and modulating inflammation might offer therapeutic perspectives for autoimmune diseases. In the present study, we investigated the role of anx A1 not only for apoptotic cell removal, but also for the still rather unknown engulfment mechanisms of secondary necrotic cells. Surprisingly, we found that externalization of anx A1 to the cell surface is a rather late event in comparison to PS exposure and occurred mainly after

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1 Address correspondence and reprint requests to Kirsten Lauber, Department of Internal Medicine I, Eberhard-Karls-University, Otfrid-Mueller-Str. 10, Tuebingen, Germany. E-mail address: kirsten.lauber@med.uni-tuebingen.de

2 Abbreviations used in this paper: PS, phosphatidylserine; PI, propidium iodide; Anx, annexin; siRNA, small interfering RNA.

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the transition from apoptosis to secondary necrosis. We also demonstrate that the opsonization of apoptotic cells with soluble recombinant anx A1 strongly facilitates their phagocytic uptake. In contrast, the engulfment of secondary necrotic cells occurred independently of anx A1, suggesting that the removal of apoptotic and secondary necrotic cells is mediated by distinct pathways. Intriguingly, despite the failure to affect the uptake of secondary necrotic cells, anx A1 efficiently prevented the secretion of proinflammatory cytokines in macrophages after ingestion of secondary necrotic cells. Thus, our results suggest that anx A1 exposure provides an important anti-inflammatory mechanism, not only by promoting the removal of apoptotic cells, but also by preventing proinflammatory cytokine production after the transition from apoptosis to secondary necrosis.

Materials and Methods

Cell lines
Jurkat and THP-1 cells (both from ATCC) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml of penicillin, 0.1 mg/ml streptomycin, and 10 mM HEPES (all from Invitrogen Life Technologies). Cells were grown at 37°C in a 5% CO2 atmosphere and maintained in log phase. For macrophage differentiation 1 × 10⁶ THP-1 cells per well were seeded into 24-well plates, treated with 10 nM PMA (Sigma-Aldrich) for 16 h and cultivated for further 48 h.

Abs, small interfering RNA (siRNA) oligonucleotides, and other reagents
Stauroporine was purchased from Roche Molecular Biochemicals, the agonistic anti-CD95 Ab CH11 from Medical Biological Laboratories, and etoposide from Sigma-Aldrich. The following mAbs were used for flow cytometry and immunoblot analyses: anti-annexin A1, anti-annex A2, anti-annex A7 (all from BD Biosciences), and anti-vinculin (Sigma-Aldrich). Chemically modified siRNA oligonucleotides (stealth RNAi) were purchased from Invitrogen Life Technologies. The following sequences were used: anx A1 nt148: 5′GCC UUG CAU AAG GCC AUA AUG GUU A3′, anx A1 nt993: 5′ GGA UUA UGG UUU CCC GUU CUG AAA U3′, anx A1 scramble: 5′GGA UAG GUU CCC GUU CUG AAA UUA U3′.

Induction of apoptosis and secondary necrosis
Two × 10⁶ or 1 × 10⁶ cells per well were cultured in 6-well or 96-well plates, respectively. Apoptosis was induced by incubating cells with 2.5 μM stauroporine, 25 μg/ml etoposide, or 100 ng/ml anti-CD95 Ab for the indicated times. Alternatively, cells were UV-irradiated with 10 mJ/cm² in the UV Stratalinker 2400 (Stratagene) as described previously (13).

Electroporation with siRNA oligonucleotides
Electroporation with siRNA oligonucleotides was conducted twice (at day 0 and day 3) with the Gene Pulser II plus Capacity Extender II (BioRad) and 0.4 cm gap cuvettes as described previously (14). In brief, 5 × 10⁶ Jurkat cells were electroporated with 2 μM of siRNA oligonucleotides in 500 μl OptiMEM medium (Invitrogen Life Technologies) by a single pulse (800 μF, 200 V, time constant 20–30 ms). The cells were cultured for 3 days before electroporation was repeated. All following experiments were conducted at day 6.

FACS staining of annexins and vinculin
For surface staining of different annexins and vinculin on native cells, cells were resuspended in ice-cold staining buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM CaCl₂, 1% heat-inactivated human serum and 0.1% NaN₃) supplemented with 0.5–2 μg/ml primary Ab and incubated for 30 min on ice. After two washing steps in staining buffer cells were incubated with a 1/500 dilution of Cy2-labeled secondary Ab (GE Healthcare) in staining buffer for 30 min on ice. After two further washing steps cells were resuspended in staining buffer supplemented with 2 μg/ml propidium iodide (PI) and analyzed by flow cytometry on a FACSCalibur (BD Biosciences). For staining of permeabilized cells (Fig. 3B), the Cytofix/Cytoperm kit (BD Biosciences) was used according to the manufacturer’s recommendations.

Surface staining with Cy2-labeled anx A1 mutants
Five × 10⁵ cells per well were washed in staining buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM CaCl₂) and incubated with 0.2 nM of the different Cy2-labeled anx A1 mutants diluted in 100 μl staining buffer supplemented with 2 μg/ml PI for 5 min on ice. Cells were collected by centrifugation, washed in staining buffer, and analyzed by flow cytometry.

Flow cytometric measurement of plasma membrane integrity, PS externalization, and DNA fragmentation
Plasma membrane integrity was determined flow cytometrically by exclusion of PI. Cells were stained in PBS supplemented with 2 μg/ml PI. Cells positive for PI staining were considered to have a leaky plasma membrane. Externalization of PS was measured by staining with annexin A5-FITC/PI (annexin A5 FLUOS staining kit, Roche) and subsequent flow cytometric analysis. Cells with positive annexin A5-FITC but negative PI staining were considered apoptotic, whereas cells double-positive for annexin A5-FITC and PI staining were considered secondary necrotic. For determination of DNA fragmentation the percentage of sub G1 nuclei was measured by flow cytometry as described before (15).

Expression, purification, and labeling of recombinant anx A1
Escherichia coli BL21 (DE3) expression clones of histidine-tagged human anx A1 (aa 1a–46, aa 1a–346, and aa 47–346) in the plasmid pET15b were generated according to standard cloning procedures and His-tagged proteins were purified as described previously (15). Afterwards, the His-tag was cleaved off by incubation with immobilized thrombin (Merck) according to the manufacturer’s instructions. LPS was removed by passing the purified proteins over an Endotrap column (Protos). Purity and integrity were checked by SDS-PAGE and subsequent Coomassie staining. The purified anx A1 proteins were Cy2-labeled with the FluoroLink-Ab Cy2-labelling kit (GE Healthcare) according to the manufacturer’s instructions. In brief, 1 μg of recombinant protein was incubated with the Cy2-labelling kit for 10 min on ice in coupling buffer (1 M sodium carbonate (pH 9.3)) for 30 min at room temperature. Afterwards, non-coupled dye was separated from the labeled protein by gel filtration. Labeling was confirmed by SDS-PAGE.

Western blot analysis
Western blot analysis was performed as described previously (16) with mAbs against anx A1, anx A2, anx A7 (all from BD Biosciences), and vinculin (Sigma-Aldrich).

Measurement of cytokine production
For the determination of cytokine production, PMA-differentiated THP-1 cells (1 × 10⁶ cells per well in 24-well plates) were fed at different ratios with secondary necrotic prey cells for 2 h. Subsequently, non-engulfed prey cells were washed away and THP-1 cells were further cultivated in medium supplemented with 100 ng/ml LPS (Sigma-Aldrich) for 24 h. Cell-free supernatants were collected and cytokine concentrations (IL-1β, TNF-α, IL-6, IL-12p70, and IFN-γ) were determined with DuoSet ELISA kits (R&D Systems) according to the manufacturer’s instructions.

Phagocytosis assay
Prey cells were stained with PKH26 (Sigma-Aldrich) according to the manufacturer’s instructions. UV irradiated with 10 μJ/cm², and incubated for the indicated times. Subsequently, apoptotic or postapoptotic cells were added to 1 × 10⁶ PKH67-stained, PMA-differentiated THP-1 macrophages per well in 24-well plates in ratios from 1:1 to 4:1 (prey: phagocyte) 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 internalized prey cells (double-positive for PKH26 and PKH67 staining) on the basis of all prey cells deployed (positive for PKH26 staining). In Figs. 1B and 4B, 2.5 × 10⁵ prey cells were pretreated for 30 min at 4°C with 1 nM of the indicated proteins in 500 μl TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) supplemented with 5 mM CaCl₂. Afterwards, cells were collected by centrifugation and washed to remove unbound proteins, before the prey cells were added to the macrophages. In Fig. 4C, prey cells were washed with TBS containing 10 mM EGTA or 5 mM CaCl₂ before incubation with macrophages.

Adhesion assay
Prey cells were stained with PKH67 (Sigma-Aldrich). UV-irradiated with 10 μJ/cm² and incubated for 12 h. In brief, 2.5 × 10⁵ prey cells were pretreated with 1 nM of the indicated proteins in 500 μl TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) supplemented with 5 mM CaCl₂ for 30 min at 4°C. Afterwards, cells were collected by centrifugation and unbound proteins were washed away before the prey cells were added to 5 × 10⁶ PMA-differentiated THP-1 macrophages (ratio 4:1) in

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serum-free medium. After 30 min of incubation at 37°C unbound prey cells were carefully washed away in serum-free medium. Bound prey cells were detached in PBS containing 10 mM EDTA, collected by centrifugation, and lysed in 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, 10% glycerol, and 1% Triton X-100. PKH67 fluorescence was assessed with a FL600 fluorometer (BioTek). The number of adherent prey cells was calculated on the basis of a calibration curve and normalized to the untreated control.

FIGURE 1. Recombinant soluble anx A1 promotes the phagocytosis of apoptotic cells. A, Apoptotic Jurkat cells bind Cy2-labeled anx A1 and an anx A1 core fragment. Upper left, Domain structure of anx A1. Arabic numbers depict the amino acid position and annexin repeats are numbered I-IV. Lower left, Jurkat cells were left untreated (control) or stimulated with staurosporine for 4 h (apoptotic). Subsequently, cells were stained with purified Cy2-labeled anx A1 mutants (aa 1–346, aa 1–46, and aa 47–346) and analyzed by flow cytometry. Representative histograms of triplicates are shown (analysis gate was set on PI-negative cells). Right, SDS-PAGE of fluorescent anx A1 mutants. Five micrograms of the indicated Cy2-labeled anx A1 mutants were separated by 15% SDS-PAGE. Green Cy2-fluorescence was digitally replaced by gray staining for a better contrast. B, Coating with purified anx A1 aa 1–346 and aa 47–346 enhances phagocytic uptake of apoptotic Jurkat cells. Left, Cells were labeled with PKH26, incubated for 12 h, and added at the indicated ratios to PKH67-labeled, PMA-differentiated THP-1 macrophages. Subsequently, cells were added to PKH67-labeled, PMA-differentiated THP-1 macrophages as described in B. The percentage of internalized prey cells was determined by flow cytometry and is given as mean values ± SD from quadruplicates. Right, Apoptosis induction was verified by measurement of phosphatidylserine exposure and plasma membrane integrity using anx A5-FITC/PI staining. Representative dot plots of quadruplicates are shown. C, Soluble anx A1 promotes the phagocytosis of apoptotic THP-1 cells. Left, Cells were labeled with PKH26, and then stimulated to undergo apoptosis by UV irradiation (10 mJ/cm²), incubated for 4 h, and coated with different forms of anx A1 or BSA (1 nmol purified protein per 2.5 × 10⁶ cells). Subsequently, cells were added to PKH67-labeled, PMA-differentiated THP-1 macrophages as described in B. The percentage of internalized prey cells was determined by flow cytometry and is given as mean values ± SD from quadruplicates. Right, Apoptosis induction was verified by measurement of PS exposure and membrane integrity as described in B. D, Coating with anx A1 aa 1–346 and aa 47–346 increases the binding of apoptotic cells to macrophages. THP-1 prey cells were labeled with PKH67, stimulated to undergo apoptosis, and coated with different forms of anx A1 or BSA (1 nmol purified protein per 2.5 × 10⁶ cells) as described in C. Subsequently, cells were added to PKH67-labeled, PMA-differentiated THP-1 macrophages as described in B. The percentage of internalized prey cells was determined by flow cytometry and is given as mean values ± SD from quadruplicates. Adhesion was normalized on protein-free control samples and is given as mean ± SD of octuplicates. p values were calculated in comparison to BSA-containing samples by unpaired heteroscedastic Student’s t test analysis. *, p < 0.05.
Results

Exogenous anx A1 promotes phagocytosis of apoptotic cells

Previous studies demonstrated that anx A1 is recruited from the cytoplasm to the cell surface of dying cells where it promotes their phagocytic uptake as a bridging protein for PS (9, 10). To investigate the role of anx A1 for the engulfment of apoptotic cells in our system, we used recombinant soluble anx A1 as well as different deletion mutants, such as an N-terminally truncated version (aa 47–346) comprising only the annexin core region (Fig. 1 A). Because certain biological activities of anx A1 are mediated by its N-terminus (17), we also included an N-terminal fragment (aa 1–46) lacking the PS-binding annexin boxes in our analyses. For flow cytometric analysis, full-length anx A1 and the mutants were labeled with Cy2 fluorescent dye and then incubated with Jurkat cells that had been rendered apoptotic by incubation with staurosporine. Apoptotic Jurkat cells revealed a strong binding of recombinant full-length anx A1 as well as of the mutant harboring the core region (Fig. 1 A). Anx A1 binding was also observed in other apoptotic cell types, such as THP-1 monocytes, and in response to different apoptotic stimuli including UV-irradiation (supplementary Fig. 1). Control cells, however, did not bind anx A1, presumably reflecting a strong PS exposure in apoptotic but not healthy cells. In addition, the N-terminal fragment (aa 1–46) lacking the PS binding sites was inefficient to bind to apoptotic cells (Fig. 1 A and supplementary Fig. 1).

Exogenous anx A1 did not only bind to apoptotic cells, but also efficiently promoted their uptake by PMA-differentiated THP-1 macrophages that were used as phagocytes in the experiment. As shown in Fig. 1B, apoptotic prey cells that had been left untreated

5 The online version of this article contains supplemental material.
or coated with the N-terminal (aa 1–46) anx A1 fragment were only weakly taken up by THP-1 macrophages, whereas cells coated with full-length anx A1 or the aa 47–346 mutant were efficiently engulfed.

Control measurements of PS exposure and membrane integrity ensured that the prey cells employed were primary apoptotic with >70% PS-single-positive and <10% PS/PI-double-positive cells (Fig. 1B). Coating with anx A1 also strongly enhanced the engulfment of apoptotic THP-1 prey cells (Fig. 1C) suggesting that the effect of anx A1 was not cell type-specific. The enhanced uptake of anx A1-coated prey cells was presumably caused by an improved attachment to the phagocyte, as revealed by a significantly augmented adhesion of prey cells opsonized with full-length anx A1 or the core mutant in comparison to BSA-treated cells (Fig. 1D). These observations are consistent with previous notions that anx A1 acts an important bridging protein for apoptotic cell removal. Moreover, our data indicate that the N-terminus devoid of the PS-binding annexin repeats is dispensable for this function of anx A1.

The majority of anx A1 externalizing cells is secondary necrotic

Since the initial reports on anx A1 externalization did not distinguish between apoptosis and secondary necrosis (9, 10), we next investigated the time course of anx A1 exposure during cell death. To this end, Jurkat cells were stimulated with staurosporine for 0–12 h, and anx A1 externalization was assessed by immunostaining in combination with PI exclusion. Surprisingly, the majority of anx A1-positive cells turned out to be already PI-positive and, hence, secondary necrotic, whereas only a small proportion of cells with exposed anx A1 (max. 10%) was early apoptotic and still retained an intact plasma membrane (Fig. 2A).

To substantiate these unexpected findings, we measured anx A1 externalization in parallel with PI exclusion and PS externalization, which indeed revealed that PS exposure preceded PI uptake and anx A1 externalization by several hours (Fig. 2B). This observation was further supported by a simultaneous triple staining of PS exposure, anx A1 externalization and membrane permeabilization as assessed by the uptake of 7-AAD. A time course analysis demonstrated an early PS exposure, while anx A1 externalization was only detectable, when the majority of cells already had a leaky plasma membrane and hence stained positive for 7-AAD (supplementary Fig. 2). Apart from staurosporine, other cell death inducers, such as anti-CD95 Ab, etoposide, and UV-irradiation instigated the externalization of anx A1, however, with even more delayed kinetics (Fig. 2C), although all agents potently induced cell death as assessed by flow cytometric measurement of DNA fragmentation (Fig. 2D). To substantiate that anx A1 externalization was not restricted to Jurkat cells, we also examined primary PHA-stimulated lymphoblasts, monocytes, and neutrophils. Albeit to a different extent, all cell types displayed externalization of anx A1 after staurosporine treatment in the stage of secondary necrosis (supplementary Fig. 3). Thus, in contrast to PS exposure, externalization of anx A1 does not represent an early event during apoptosis, but rather occurs at a late stage of postapoptotic, secondary necrosis.

Externalization during cell death is not a general feature of the annexin family

Next, we tested whether other members of the annexin family were externalized as well. Jurkat cells were induced to undergo secondary necrosis with different cell death stimuli, and externalization of anx A1, A2, and A7 was measured by immunostaining. Intriguingly, externalization was only observed for anx A1, but not in the case of anx A2 and A7, indicating that it is not a general but rather a specific feature of anx A1 (Fig. 3A). Western blot analysis was employed to ensure that the Jurkat cells actually expressed anx A1, A2, and A7. Furthermore, as secondary necrotic cells have a leaky plasma membrane, we ascertained that the immunostaining was not mediated by a penetration of the Ab into the cytoplasm. In contrast to anx A1, virtually no staining was obtained for the actin-associated cytoskeletal protein vinculin, which served as a negative control (Fig. 3A), indicating that anx A1 was detected at the cell surface and not in the cytoplasm of the secondary necrotic cells. As a positive control, fixed and permeabilized cells were also analyzed demonstrating positive staining signals for all annexins and vinculin, regardless of whether vital or secondary necrotic cells were analyzed (Fig. 3B).

Engulfment of secondary necrotic cells is independent of anx A1

Although autoimmune diseases are known to be associated with delayed or deficient apoptotic cell removal and a consequent accumulation of secondary necrotic debris, the mechanisms of engulfment of secondary necrotic cells are largely unknown. We therefore investigated, whether, similar to the uptake of apoptotic cells, also the engulfment of secondary necrotic cells is promoted
FIGURE 4. Phagocytosis of secondary necrotic cells occurs independently of anx A1. A, Induction of secondary necrosis. Jurkat cells were stimulated to undergo secondary necrosis by UV-irradiation (10 mJ/cm²). After 18 h, PS externalization and plasma membrane integrity were measured. Representative dotplots of quadruplicates are shown. B, Coating with purified anx A1 aa 1–346 and aa 47–346 only weakly enhances the phagocytic uptake of secondary necrotic cells. Left, Jurkat cells were labeled with PKH26 and stimulated to undergo secondary necrosis as described in A. Subsequently, cells were incubated with different forms of anx A1 or BSA (1 nmol purified protein per 2.5 × 10⁶ cells) as in Fig. 1B and fed in different ratios to 1 × 10⁵ PKH67-labeled, PMA-differentiated THP-1 macrophages. After 2 h, the percentage of internalized prey cells was determined by flow cytometry and is given as mean ± SD from quadruplicates. Right, Flow cytometric analysis of the Cy2-labeled proteins revealed that anx A1 aa 1–346 and aa 47–346 efficiently bound to secondary necrotic cells. C, Removal of externalized anx by EGTA does not reduce their phagocytic uptake. Left, PKH26-stained Jurkat cells were stimulated to undergo secondary necrosis by UV-irradiation (10 mJ/cm²). Fourteen hours after irradiation, cells were washed in TBS with 5 mM CaCl₂ or 10 mM EGTA and fed to PKH67-labeled, PMA-differentiated THP-1 macrophages in different ratios. The percentage of internalized prey cells was determined by flow cytometry and is given in mean values ± SD from quadruplicates. Right, Flow cytometric analysis confirmed that washing with EGTA efficiently removed anx A1 from the cell surface. D, Knock-down of anx A1 expression does not reduce the phagocytic uptake of secondary necrotic cells by macrophages. Knock-down of anx A1 expression was conducted by electroporation of Jurkat cells with 2 different anx A1-specific oligonucleotides and a scramble control oligonucleotide. On day 5 after the first electroporation the cells were stained with PKH26 and stimulated to undergo secondary necrosis. Left, Prey cell internalization was determined by flow cytometry and is given as mean values ± SD from quadruplicate experiments. Right, Immunoblot analysis of anx A1-silenced or scramble control Jurkat cells confirmed the efficient down-regulation of anx-1 expression but not the control protein vinculin. E, Externalization of anx A1 is reduced in anx A1 knock-down cells. Anx A1-silenced or scramble control Jurkat cells were UV-irradiated and incubated for the indicated times. Externalization of anx A1 was measured as in Fig. 2A and is displayed as the percentage of anx A1-positive cells (left) or relative anx A1 externalization (median Fl-1 signal of anti-anx A1 staining relative to the isotype control; right). Mean values ± SDs of triplicates are shown.
by anx A1. To this end, Jurkat cells were stimulated to undergo secondary necrosis after prolonged incubation post irradiation (Fig. 4A). Similar to the previous experiments with apoptotic cells (Fig. 1), the secondary necrotic cells were then loaded with different versions of purified anx A1 and added to macrophages. However, in marked contrast to the uptake of apoptotic cells, the engulfment of secondary necrotic cells was only weakly enhanced by exogenous anx A1 (Fig. 4B), although anx A1 was efficiently coated on the cells (Fig. 4B).

Since exogenous anx A1 had only minor effects, we next examined the effect of endogenous anx A1 on the ingestion of secondary necrotic cells. To this end, we used two different approaches. First, we removed surface-bound anx A1 by washing the secondary necrotic Jurkat cells with the calcium chelator EGTA, fed them to macrophages, and then measured their internalization. In comparison to prey cells, which had been washed in the absence of EGTA, we did not detect a significant reduction in the phagocytic uptake of EGTA-washed cells (Fig. 4C). In a second approach, we down-regulated anx A1 expression by RNA interference using two different anx A1-specific oligonucleotides. Although immunoblot analysis confirmed the successful knockdown of anx A1, no decrease in the uptake of anx A1-silenced cells was detected in comparison to scramble control cells (Fig. 4D). As expected, silencing of anx A1 expression led to its reduced membrane exposure during secondary necrosis (Fig. 4E). These results therefore imply that neither down-regulation nor removal of endogenous anx A1 from the surface of secondary necrotic cells interferes with their engulfment.

**Secondary necrotic anx A1 knock-down cells exhibit an enhanced proinflammatory potential**

The previous results demonstrate that coating with purified anx A1 supports the engulfment of apoptotic cells, but hardly affects the uptake of secondary necrotic cells. Consequently, the question arises, which function is exerted by anx A1 on the surface of secondary necrotic cells. Anx A1 has been reported to mediate...
diverse anti-inflammatory effects (12), but its role in the postphagocytic macrophage reaction has so far not been addressed. Since secondary necrotic cells, unlike apoptotic cells, stimulate a proinflammatory response in phagocytes (3), we therefore analyzed the cytokine production by macrophages in response to the engulfment of secondary necrotic anx A1 knock-down or scramble control cells. Overall, macrophages, which had ingested increasing numbers of secondary necrotic cells, dose-dependently released the proinflammatory cytokines IL-1β, TNF, and IL-6, whereas the production of IL-12p70 and IFN-γ remained unaffected (Fig. 5A). Most importantly, secondary necrotic cells, in which anx A1 expression had been knocked down, stimulated a significantly stronger production of IL-1β, TNF, and IL-6 than scramble control cells (Fig. 5A). This increase in cytokine production was not due to an influence of anx A1 knock-down on the general outcome of secondary necrosis (Fig. 5B). Thus, externalization of anx A1 efficiently attenuates the proinflammatory nature of secondary necrotic cells.

**Soluble anx A1 inhibits proinflammatory cytokine production by macrophages**

To test, whether the reduced secretion of IL-1β, TNF, and IL-6 in response to anx A1 externalization can be mimicked by purified anx A1 alone, we measured the cytokine production by LPS-stimulated macrophages in the absence or presence of exogenous anx A1. We observed a profound and dose-dependent inhibition of IL-6 release by full-length anx A1. The inhibitory effect on IL-1β and TNF production was weaker but still significant (Fig. 6). Interestingly, a significant inhibition of cytokine production was only detected upon addition of the full-length form of anx A1. Addition of the N-terminal anx A1 fragment or the core domain did not reduce IL-6 or IL-1β production, and even slightly increased TNF secretion. Altogether, our results indicate that anx A1 exposure during secondary necrosis provides an important anti-inflammatory failsafe mechanism, even when the clearance of apoptotic cells fails.

**Discussion**

Disturbances in apoptotic cell removal and the concomitant onset of secondary necrosis are known to promote the development of chronic inflammation and autoimmunity. One possible approach toward a molecular targeted therapy for autoimmune diseases including systemic lupus erythematous is therefore enhancement of the phagocytic uptake of apoptotic and secondary necrotic cells. Promising target molecules in this regard are bridging proteins of the phagocytic synapse including anx A1, which is recruited from the cytoplasm to the cell surface and thereby can opsonize apoptotic cells for phagocytosis. In the present study, we show that anx A1 does not only promote the phagocytic uptake of apoptotic cells, but in addition prevents inflammation by inhibiting proinflammatory cytokine production in macrophages, which have ingested secondary necrotic cells. Our results indicate that this dual activity of anx A1 may provide an important failsafe mechanism for preventing chronic inflammation and autoimmunity.

Previous studies reported the externalization of endogenous anx A1 during cell death and its role as a PS-binding bridging protein in the phagocytic synapse (9, 10). Consistent with these findings, we now demonstrate that also coating of apoptotic cells with soluble recombinant anx A1 promotes their phagocytosis. Using different mutants of anx A1, our results for the first time show that solely the PS-binding sites of anx A1 are required for this activity, since opsonization with the full-length protein or the core domain but not the N-terminal anx A1 fragment promoted the uptake of apoptotic cells. That the core domain alone was sufficient for phagocytosis promotion is surprising but might be explained by a bridging of externalized PS on the dying cell with PS on the macrophage surface (18). Intriguingly, it was reported that N-terminal peptides of anx A1 can bind to members of the formyl-peptide receptor family on the macrophage cell surface and thereby can promote phagocytosis of apoptotic cells (17). Our results therefore suggest that different domains of anx A1 may trigger different activities in cis or trans depending on the target cell.

Consistent with previous studies (9, 10), we report that externalization is rather specific for anx A1, as the close relatives anx A2 and anx A7 remained cytoplasmic and were not exposed during cell death. Surprising is however our finding that externalization of anx A1 represents a rather late event. In a detailed time course analysis, we found that the externalization of anx A1 was strongly delayed in comparison to PS exposure and mostly detected in cells that had already lost their membrane integrity. It should be mentioned that the kinetics of anx A1 exposure has not been investigated so far. Previous studies, although employing the same cell types (Jurkat cells and primary PHA-stimulated lymphoblasts) as well as identical or similar apoptosis inducers (CD95 ligation, treatment with camptothecin or UV irradiation), did either not combine anx A1 surface staining with an analysis for plasma membrane integrity, or excluded secondary necrotic cells from the flow cytometric analyses (9, 10). Thus, our data clearly indicate that anx A1 externalization is a late event in the course of apoptosis, taking place in a phase in which most of the cells are already secondary necrotic. If apoptotic cells per se do not externalize anx A1 the question about the physiological relevance of anx A1-aided apoptotic cell engulfment arises. From our data and the fact that macrophages and other cell types can secrete anx A1 constitutively or upon stimulation (10, 19–21), it is conceivable that macrophages...
might actively coat apoptotic cells with anx A1 to facilitate their phagocytic uptake.

Although the uptake of apoptotic cells has been intensively studied, the mechanisms of engulfment of primary and secondary necrotic cells are largely unknown. We therefore investigated the role of anx A1 in the uptake of secondary necrotic cells. Although we found that anx A1 efficiently promoted the uptake of apoptotic cells, several lines of evidence clearly indicate that the engulfment of secondary necrotic cells occurred independently of anx A1. Thus, addition of recombinant anx A1 only weakly promoted the uptake of secondary necrotic cells. Furthermore, removal of endogenous anx A1 from the cell surface using calcium chelation did not alter the uptake. Finally, we demonstrate that RNAi-mediated knock-down of anx A1 expression did not impair the internalization of secondary necrotic cells. These results therefore suggest that the engulfment of apoptotic and secondary necrotic cells is controlled by distinct mechanisms. Future studies have to show, whether, among the various “eat-me” signals and bridging molecules involved in apoptotic cell recognition, other regulators mediate the uptake of secondary necrotic cells (1).

A major finding of this study is that, despite its failure to control phagocytosis of secondary necrotic cells, anx A1 exposure exerts a potent anti-inflammatory effect on macrophages following the ingestion of secondary necrotic cells. Anx A1 is considered as a crucial mediator of anti-inflammatory glucocorticoid action (12), but its role in the postphagocytic macrophage reaction has so far not been addressed. It is generally assumed that, in contrast to apoptotic cells, the uptake of secondary necrotic cells exerts a proinflammatory effect by triggering cyto-
kine expression (3, 22). Indeed, our experiments demonstrate that macrophages respond to the ingestion of secondary necrotic cells with a dose-dependent secretion of IL-1β, TNF, and IL-6. Most importantly, the amount of proinflammatory cytokines released by macrophages was significantly increased upon ingestion of secondary necrotic cells with silenced anx A1 expression. These results suggest that suppression of proinflam-

atory cytokine production is an important function of anx A1 exposed on the surface of secondary necrotic cells and, thus, add a new facet to the previously known anti-inflammatory ef-

fects of anx A1.

Anx A1-deficient mice display an enhanced susceptibility to inflammatory stimuli and a decreased sensitivity to glucocorticoid treatment (23). Employing a model of Ag-induced arthritis augmented IL-1 and IL-6 mRNA levels were detected in the joints of anx A1−/− mice (24). Furthermore, peritoneal macrophages of anx A1−/− mice displayed an increased production of IL-1 and IL-6 in response to LPS, culminating in a strongly increased lethality to septic shock (25). Intriguingly, the effects of anx A1 on the phago-
cytosis of apoptotic cells and postphagocytic cytokine production are rather opposite to activities described for anx A5. Several studies showed that coating with anx A5 inhibits the uptake of apop-
totic cells and promotes proinflammatory cytokine production (26–29). Moreover, anx A1 and anx A5 knock-out mice display distinct phenotypes regarding inflammation (23, 24, 30). The molecular basis of these differences is unknown and an interesting subject of future studies.

The mechanisms by which anx A1 mediates its anti-
inflammatory effects are far from being understood. It is conceivable that externalized anx A1 on the dead cell surface directly binds to receptors on the phagocyte and thereby interferes with cytokine production. Our data support this notion, since addition of purified anx A1 strongly reduced LPS-induced IL-6 production and, albeit to a lesser extent, also IL-1β and TNF secretion. Inter-

estingly, prevention of cytokine production by anx A1 required the full-length protein, whereas the N-terminal anx A1 fragment was unable to inhibit cytokine secretion. Surprisingly and in contrast to its ability to promote apoptotic cell engulfment, the core domain also failed to inhibit cytokine secretion, suggesting that different parts of the anx A1 molecule are involved in the control of phago-
cytosis and cytokine production. It has been described that anx A1 inhibits neutrophil extravasation by binding to members of the formyl-peptide receptor family (31–33). This anti-inflammatory activity, which presumably involves receptor desensitization, is interestingly retained in the N-terminal fragment of anx A1. Since in our study the N-terminus alone did not inhibit cytokine produc-
tion in response to ingestion of secondary necrotic cells, receptors other than formyl-peptide receptors are presumably required for this effect.

Our results suggest that externalization of anx A1 by dying cells and the concomitant inhibition of postphagocytic IL-1β, IL-6, and TNF production represent a final counter measure to ameliorate the proinflammatory milieu of secondary necrosis. This observation and the beneficial effects of purified anx A1 on the phagocytosis of apoptotic cells render anx A1 an interesting target molecule for future therapies of inflammatory disorders associated with defective clearance of dying cells. It should be noted that local administration of anx A1 or N-terminal peptides has already successfully been employed to alleviate various types of inflammatory reactions in mouse models (34–36).

From our results it can be concluded that based on its dual activity, namely promoting the clearance of apoptotic corpses and preventing cytokine expression after the uptake of second-
ary necrotic cells, exposure of anx A1 provides an efficient failsafe mechanism counteracting inflammatory responses, even when the timely removal of apoptotic cells has failed.

Disclosures
The authors have no financial conflict of interest.

References

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