# The role of annexin A1 in secondary necrosis

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The author of this thesis performed the majority of experiments (Fig 1, 2B, 3B, 4A, B, E; 5, 6 and supplemental figures) and contributed to write the manuscript. The kinetic analysis of anx A1 externalization, detection of anx A1 externalization after treatment with different stimuli including the measurement of sub G1 nuclei (Fig. 2 A, C and D), and the analysis of externalization of other annexins (Fig. 3A) were done by S.S. The phagocytosis assays with EGTA treated secondary necrotic cells and anx A1 knock down cells (Fig. 4C, D) were performed by M. W.. The analysis of the knock down efficiency of anx A1 on mRNA level, which was not included in the final version of the publication, was done by H. K. which were not included in the final version of the publication. S.W., M.H. and K.S.-O. contributed to write the manuscript. K.L. supervised and designed research and wrote the manuscript.

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# 1. Zusammenfassung / Summary

## 1.1 Zusammenfassung: Die Rolle von Annexin A1 in der sekundären Nekrose

In mehrzelligen Organismen sterben täglich mehrere Milliarden Zellen durch den programmierten Zelltod Typ1, Apoptose, wodurch eine Aufrechterhaltung der Gewebshomöstase gewährleistet wird. Die sterbenden und toten Zellen werden durch professionelle Phagozyten beseitigt, ohne das Immunsystem zu aktivieren. Eine Vielzahl an Signalmolekülen ist an der Erkennung und Eliminierung sterbender Zellen beteiligt. Diese werden anhand ihrer Funktion in 'find-me', und 'eat-me' Moleküle, Brückenproteine und ihre entsprechenden Rezeptoren unterteilt werden. Das koordinierte Zusammenspiel dieser Signalmoleküle ist die Voraussetzung für die rechtzeitige Beseitigung apoptotischer Zellen. Werden apoptotische Zellen durch eine Störung nicht rechtzeitig eliminiert, dann gehen sie in das Stadium der sekundären Nekrose über. Diese wiederum ist charakterisiert durch die Freisetzung des intrazellulären Inhalts auf Grund der fortscheitenden Durchlässigkeit der Zellmembran und kann zur Induktion von chronischen Entzündungen und / oder Autoimmunerkrankungen führen. Die Ursachen für das Auftreten sekundärer Nekrose können unterschiedlicher Natur sein. Störungen bei der Eliminierung apoptotischer Zellen oder massive Apoptoseinduktion, die die verfügbare Phagozytosekapazität überschreitet, können eine entscheidende Rolle spielen.

In der vorliegenden Arbeit konnte gezeigt werden, dass das Brückenprotein Annexin A1 (anx A1), das bereits im Zusammenhang mit verschiedenen immunologischen Prozessen beschrieben wurde, bei der Eliminierung sekundär nekrotischer Zellen und der anschließenden Immunantwort eine wichtige Rolle spielt.

Im ersten Teil dieser Arbeit sollte die Rolle von Annexin A1 (anx A1) im Zusammenhang mit der Phagozytose sterbender Zellen und der post-phagozytären Immunantwort untersucht werden. Anx A1 ist ein intrazelluläres Protein, das mit der effizienten Eliminierung apoptotischer Zellen in Verbindung gebracht wird. Übereinstimmend mit seiner Funktion als Brückenmolekül in der phagozytotischen Synapse konnten wir zeigen, dass die Erkennung apoptotischer Zellen durch die exogene Zugabe von gereinigtem anx A1 die Aufnahme durch professionelle Phagozyten deutlich verstärkte. In einer detaillierten Analyse stellte sich heraus, dass anx A1 nur schwach von apoptotischen Zellen aber sehr stark von sekundär nekrotischen Zellen auf die Zelloberfläche externalisiert wurde. Die Phagozytose sekundär nekrotischer Zellen wurde durch Zugabe von gereinigtem anx A1 jedoch nicht verstärkt. Interessanterweise, konnte mit dieser Arbeit gezeigt werden, dass die Externalisierung von anx A1 auf der Zelloberfläche von

sekundär nekrotischen Zellen die post-phagozytäre Immunantwort von Macrophagen beeinflusste. Es wurde eindeutig nachgewiesen, dass nach Aufnahme sekundär nekrotischer Zellen durch Macrophagen, diese in Anwesenheit von anx A1 deutlich weniger proinflammatorische Zytokine freisetzten als in Abwesenheit von anx A1 auf der sekundär nekrotischen Zelloberfläche.
Die Ergebnisse im ersten Teil dieser Arbeit zeigten, dass die Externalisierung von anx A1 während der sekundären Nekrose einen wichtigen Notfallmechanismus darstellen, der Entzündungsreaktionen entgegen wirkte, gerade dann wenn die rechtzeitige Aufnahme apoptotischer Zellen nicht gewährleistet war.
Im zweiten Teil der vorliegenden Arbeit, konnten wir zu Beginn beobachten, dass die Externalisierung von anx A1 nach Induktion der sekundären Nekrose nur in bestimmten Zelltypen erfolgte, und mit einer proteolytischen Prozessierung von anx A1 auf der Zelloberfläche einherging. In den folgenden Untersuchungen wurde die Metalloprotease ADAM10 als diejenige Ectoprotease identifiziert, die anx A1 in seiner einzigartigen N-terminalen Domäne prozessierte. Dies führte zur Freisetzung eines kleinen Peptides von der Oberfläche sekundär nekrotischer Zellen, das in der Lage war, THP-1 Monozyten zu rekrutieren. Diese Beobachtung konnte durch eine deutlich reduzierte Transmigration auf Zellkulturüberstände sekundär nekrotischer Zellen, in denen die anx A1 oder die ADAM10 Expression inhibiert wurde, bestätigt werden.
Die Externalisierung und die proteolytische Prozessierung von anx A1 stellen damit ein finales Ereignis während der Apoptose dar, das nach dem Übergang in die sekundäre Nekrose zur Anlockung von Monozyten beiträgt.
Anx A1 hat daher offensichtlich mehrere Funktionen bei der Entsorgung sterbender Zellen: (I) als 'find-me' Signal, das von sekundär nekrotischen Zellen freigesetzt wird, (II) als 'eat-me' Signal, das die Bindung zwischen sterbender Zelle und Phagozyt unterstützt und (III) eine 'anti-inflammatorische' Funktion bei der Beseitigung sekundär nekrotischer Zellen. Da Defekte in der Eliminierung apoptotischer Zellen eine entscheidende Rolle bei der Entstehung bestimmter Autoimmunerkrankungen spielen, eröffnen diese neu beschriebenen Funktionen von anx A1 potentielle Perspektiven für die Entwicklung neuer Therapien von Autoimmunerkrankungen oder chronisch entzündlichen Prozessen.

## 1.2 Summary: The role of annexin A1 in secondary necrosis

Everyday, in multicellular organisms several billions of cells die by programmed cell death type 1, apoptosis, whereby tissue homoeostasis is guaranteed. Dying and dead cells are engulfed by professional phagocytes without activation of proinflammatory immune responses. A multitude of signal molecules are involved in the recognition and the

elimination of apoptotic cells. They can be divided on basis of their function into 'find-me' and 'eat-me' molecules, bridging proteins and their corresponding phagocyte receptors. The coordinated interaction of these signal molecules is a requirement for the timely removal of apoptotic cells. In case of dysfunctions in the clearance of dying cells, apoptotic cells undergo secondary (post-apoptotic) necrosis, which is characterized by release of the intracellular content due to proceeding loss of cell membrane integrity. Potentially cytotoxic and antigenic intracellular contents are able to induce proinflammatory immune responses, which can lead to the development of chronic inflammation and autoimmune diseases. The causes of the transition to secondary necrosis can be of different nature. For example, disfunctions in the elimination of apoptotic cells or high apoptosis rates, overwhelming the available scavenging capacity can play an important role.

In the present thesis, it could be shown, that the bridging protein annexin A1 (anx A1), which was previously described in the context of different immunological processes, plays an important role in the elimination of post-apoptotic secondary necrotic cells and the post-phagocytic immune response.

In the first part of this thesis, the role of anx A1 should be investigated in the context of phagocytosis of dying cells and the post-phagocytic immunological response. Anx A1 is an intracellular protein, which is known to be involved in efficient dying cell clearance. Consistent with its function as a bridging molecule in the phagocytic synapse, we could show that recognition and engulfment of apoptotic cells by professional phagocytes can be enhanced by addition of exogenous purified anx A1. In a detailed analysis we could show that anx A1 is only slightly externalized on the apoptotic cell surface but strongly exposed by secondary necrotic cells. The phagocytosis level of secondary necrotic cells could not be enhanced by addition of exogenous anx A1. Most interestingly, we could demonstrate that anx A1 externalisation on secondary necrotic cells strongly influenced the post-phagocytic response when these cells were ingested by macrophages. It could be clearly demonstrated that engulfment of secondary necrotic anx A1 externalizing cells resulted in a strongly reduced proinflammatory cytokine production in comparison to secondary necrotic cells, not exposing anx A1 on their cell surface. These results suggest that externalization of anx A1 during secondary necrosis displays an important failsafe mechanism to counteract against proinflammatory reactions, even when timely removal of apoptotic cells is impaired.

In the second part of this thesis, it could be initially observed that only certain cell types externalized anx A1 after induction of secondary necrosis, and and that anx A1 externalization was accompanied by its proteolytical cleavage. In further investigations,

the ‘a disintegrin and metalloprotease’ 10 (ADAM10) was identified to be responsible for anx A1 processing within its unique N-terminal domain. Thereby a small peptid of 6 amino acids was generated and released from the secondary necrotic cell surface. Most interestingly, this anx A1 N-terminal derived peptide was able to induce THP-1 monocytes recruitment. This observation was experimentally substantiated by a strong reduction of THP-1 transmigration to secondary necrotic cell culture supernatants when anx A1 or ADAM10 expression was silenced by administration of specific small interfering RNAs.

Hence, the exposure and the proteolytic cleavage of anx A1 obviously represent final events of apoptosis, which after transition to secondary necrosis lead to monocyte recruitment to the site of impaired apoptotic cell clearance.

Thus, anx A1 executes several functions in the elimination of dying cells: (I) as 'find-me' signal, released from secondary necrotic cells to recruit phagocytes, (II) as 'eat-me' signal, exposed on the cell surface to enhance binding between dying cell and phagocyte, and (III) as 'anti-inflammatory' mediator, reduced proinflammatory cytokine production after engulfment of secondary necrotic cells by the phagocyte. These newly described functions of anx A1 could have an important impact on novel therapeutic strategies for the treatment of chronic inflammatory processes and autoimmune diseases.

# 2. Introduction

## 2.1 Forms of cell death

During daily cellular turnover billions of cells die by controlled cell death (apoptosis), and the body has to remove the resulting cellular corpses. It is of elementary importance to get rid of the apoptotic cells, since otherwise apoptosis may convert to secondary necrosis. The concomitant loss of plasma membrane integrity leads to the release of intracellular contents, which can initiate and promote inflammation and autoimmunity (1-4). On the other hand, cells can die an accidental death as well, for example during hyperthermia or high pressure. The leaky plasma membrame of the cellular debris and the resulting intracellular contents have to be removed. This represents an even greater challenge for the phagocytic machinery in comparison to apoptotic cell removal (5) (Figure 2.1). In the following sections the different forms of cell death and the diverse clearance procedures will be described in more detail.

### 2.1.1 Apoptosis

The original sense of the Greek word 'apoptosis' describes the shedding of leaves in autumn and was used by Kerr et al. to describe the suicide program of eukaryotic cells (6). Apoptosis plays a leading role in embryonic development, tissue homeostasis in adult organisms and the elimination of cells that are damaged by mutations or viral infections. Moreover, apoptosis participates in the regulation of the immune system, for example during clonal deletion of autoreactive T cells. Apoptosis is a physiological and silent kind of cell death, where single cells, united cell structures or organs (for example during metamorphosis of amphibians) are eliminated without an inflammatory impact on the whole organism.

Apoptosis is characterized by different morphological and biochemical changes: Cells shrink and detach from neighboring cells. The chromatin condenses and the DNA is cleaved between nucleosomes by endonucleases. This results in DNA fragments with a length of approximately 180 base pairs or multiples of this (7). Loss of the plasma membrane asymmetry leads to exposure of phosphatidylserine (PS), a phosholipid which in living cells is exclusively located in the inner leaflet of the plasma membrane, and during apoptosis can be detected at the cell surface (8). Finally, the cell undergoes a process called 'blebbing' with the resulting formation of apoptotic bodies, small membrane-surrounded vesicles carrying the contents of the dying cell. Finally, apoptotic cells and apoptotic cell particles ('blebs') are recognized, engulfed and degraded by

neighbouring cells or professional phagocytes (9). Thereby, no intracellular proteins or metabolites are released into the surrounding tissue (10-12).

### 2.1.2 Primary Necrosis

Primary necrosis was once described as kind of passive, unorganized cell death which can be caused by complement lysis, high pressure, hypothermia, oxidative stress or lytic viral infections. This view has changed during the last years and primary necrosis has emerged to an alternate form of programmed cell death under certain conditions (13-14).

The outcome of necrosis leads to an early rupture of the plasma membrane, dilatation of cytoplasmic organelles, in particular mitochondria, and potentially cytotoxic and autoantigenic intracellular contents are released. The leaked intracellular components are associated with the induction of proinflammatory responses and act as endogenous danger signals or damage associated molecular patterns (DAMPs) that activate the immune system (15). Furthermore, necrosis has been described to play a physiological role during development (e.g. the death of chondrocytes controlling the longitudinal growth of bones) and tissue homeostasis (e. g. the death of intestinal epithelial cells) (14, 16-17).

### 2.1.3 Secondary Necrosis

If the removal process of apoptotic cells is delayed or fails, apoptotic cells undergo post-apoptotic, secondary necrosis (Figure 2.1). In healthy organisms, apoptotic cells are relatively quickly ingested by professional phagocytes so that *in vivo* secondary necrotic cells are rarely to be observed. For instance in cases of high apoptosis rates or deficiencies in the phagocytosis machinery of apoptotic cells lead to an accumulation of cellular corpses and the subsequent transition to secondary necrosis. During this process apoptotic cells lose plasma membrane integrity and release potentially cytotoxic and antigenic intracellular components which promote the induction of proinflammatory reactions and autoimmune diseases (18-19).

The release of intracellular contents and nuclear components promote the formation of immune complexes with autoantigens and the development of autoreactive antibodies against self-antigens. This leads to a break in immune tolerance against self-antigens. The accumulation of secondary necrotic cell derived material and the presence of autoantibodies against intracellular antigens, including dsDNA, mitochondrial components, or citrullinated proteins/peptides in different chronic inflammatory or autoimmune diseases, such as SLE, Sjögren’s syndrome, primary biliary cirrhosis and rheumatoid arthritis support this notion (4, 20-24).

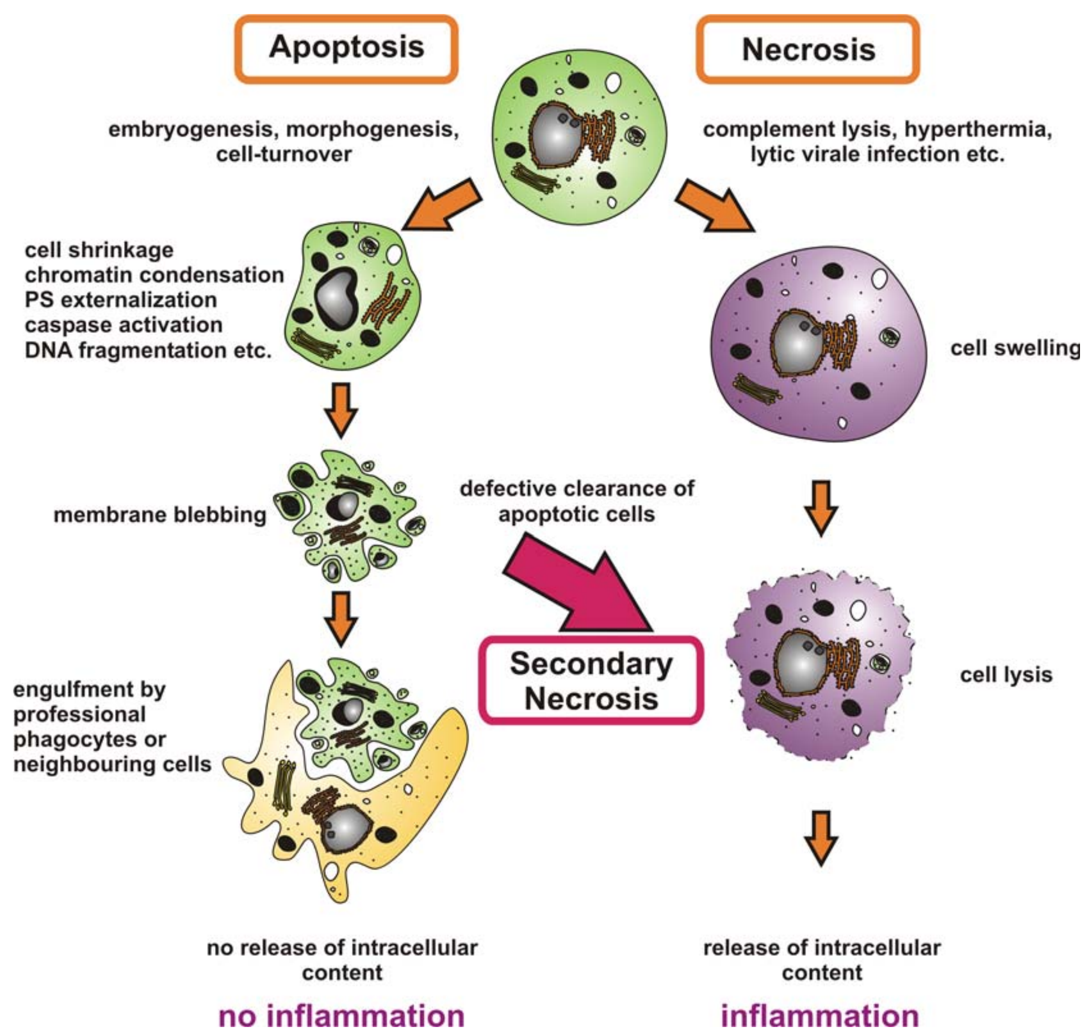


**Figure 2.1: Schematic illustration of the morphological changes during apoptosis and necrosis:** Typical characteristics for apoptotic cell death are cell shrinkage, chromatin condensation and nucleus fragmentation. Phosphatidylserine becomes externalized and finally the cell degenerates to small membrane enclosed vesicles, apoptotic bodies. They can be internalized and degraded by professional phagocytes or neighboring cells. During necrosis the cell swells, subsequently the plasma membrane ruptures and intracellular content is released. If the removal of apoptotic cells fails the plasma membrane integrity becomes compromised and apoptosis converts into secondary necrosis. Thus, non-engulfed apoptotic cells can initiate proinflammatory reactions in a comparable way as necrotic cells do.

## 2.2 Clearance of dying cells

The process of dying and dead cell clearance can be divided into three step: (I) attraction of phagocytes by soluble 'find-me' signal release, (II) recognition and engulfment of the dying cells by means of different 'eat-me' signals and finally (III) degradation of the ingested material and the post-phagocytic immune response (Figure 2.2).

### 2.2.1 Recruitment of phagocytes

Under physiological conditions dying and dead cells are engulfed by professional phagocytes or neighbouring cells. Professional phagocytes circulate in tissue and blood

vessels and have to be attracted to sites of cell death. For recruitment of phagocytes to the proximity of the cellular corpses, dying and dead cells release soluble attraction molecules - so called 'find-me' signals. The composition of soluble factors released by dying or dead cells differs between apoptotic, primary and secondary necrotic cells and apart from phagocyte recruitment contributes to the modulation of the post-phagocytic, immunological outcome (anti- or proinflammatory responses). A multitude of molecules released from different cell types have been described (15).

Apoptotic cells release 'find-me' signals which play an important role for their timely removal in order to guarantee immunogenic silence. Among others LPC, fractalkine and nucleotides (25-27) are well known 'find-me' signals that once secreted from apoptotic cells induce monocyte attraction to the site of cell death by interaction with their corresponding phagocyte receptors, including G2A, CX3CR1, and P2Y2 (27-29). The mechanisms for the release of 'find-me' molecules can be different. So far, it could be demonstrated that nucleotides are liberated via a pannexin channel (30). The chemokine fractalkine can be shedded from the cell surface by ADAM17 or ADAM10 (31). In this context, ectodomain shedding represents another important mechanism for the release of 'find-me' signals. This could be shown for the IL-6R which is released from the surface of apoptotic neutrophils by ADAM17 activity (32-33). The soluble IL-6R binds to IL-6 and the resulting complex is able to induce monocyte migration by interaction with ubiquitously expressed CD130. Other chemotactically active signal molecules liberated from apoptotic cells could be identified to play a role in recruitment of monocytes and macrophages, such as thrombospondin-1, human aminoacyl-tRNA-syntase and the S19 ribosomal protein dimer (34-35).

In contrast to apoptotic cells, necrotic cells release most of their intracellular content leading to local inflammation and alert of the innate immune system. So far only a few molecules have been identified to operate as 'find-me' signals. The most prominent molecule released from necrotic cells is HMGB-1 which has been shown to provoke immune cell reactions (36) and induce direct chemotactic attraction on neutrophils (37). Other molecules described to be released from necrotic cells are members of the S100 protein family, different heat shock proteins, uric acid, ATP, mRNA and DNA (15). They exhibit different actions on various target cells that vary from recruitment of professional phagocytes, induction of cytokine production and release to effector cell maturation.

In comparison to primary necrotic cells secondary necrotic cells have undergone the apoptotic cell death program. Hence, it could be argued that secondary necrotic cells liberate 'find-me' signals similar to those released by apoptotic cells. But one has to consider that the persistence of apoptotic 'find-me' signals is supposedly limited due to the

ubiquitous presence of degrading enzymes in the extracellular space. Furthermore, molecules released by secondary necrotic cells should also differ from primary necrotic cells because RNA is degraded, intracellular proteins are proteolytically processed, DNA is internucleosomally cleaved and ATP is consumed or released during progress of apoptosis (4, 15, 38). So far, there are only limited data available about secondary necrotic cell clearance. Uric acid was described as an endogenous danger signal liberated from both primary and secondary necrotic cells which forms extracellular monosodium urate microcrystals, that can activate the NALP3 inflammasome pathway with subsequent release of IL-1β and can induce the production of neutrophil chemotactic factors, such as IL-8 and consequently neutrophil infiltration (15, 39). Other signal molecules described for secondary necrotic cells are nucleosomes. Oligonucleosomes are formed from genomic DNA during apoptosis where free nucleosomes contain small internucleosomal DNA fragments of 180 bp or multiples of 180 bp in length (40). The oligonucleosomal DNA fragments could be detected in cytoplasma and in very late stages of apoptosis in cell-free superatants of spontaneously apoptozing lymphocytes. The authors also showed a significant proliferative and stimulatory effect on lyphocytes by released nucleosomes (41). Reports of others have identified an association of HMGB1 with secondary necrotic cell derived nucleosomes which are able to induce cytokine expression in macrophages and DC maturation (42-43).

### 2.2.2 Recognition of dying cells

After the release of soluble 'find-me' signals which leads to the recruitment of professional phagocytes to the side of dying and dead cells in higher eukaryote organisms, it is of crucial importance for the further progression of dying cell elimination that the scavenger cell recognize the cellular corpse as a corpse and internalize it afterwards. The identification of the dead cell by the phagocyte is mediated via so called 'eat-me' signals or bridging proteins, which are presented on the cell surface of the dying cells. The phagocytic synapse has developed around the multitude of membrane associated 'eat-me' signals and bridging proteins as well as various phagocytic receptors to guarantee the efficient removal of apoptotic cells (2). 'Eat-me' signals such as phosphatidylserine (PS) are externalized during cell death or they are formed by oxidation or other modifications of surface molecules.

So far, the best characterized 'eat-me' signal of apoptotic cells is PS. Translocation of PS from the inner to the outer leaflet of the plasma membrane is an important marker for the induction of apoptosis (8). The mechanisms that have been proposed to underly PS externalization during apoptosis include the inactivation of the aminophospholipid-

translocase which in living cells transports PS from the outside to the inside, the activation of lipid-scramblases, which randomize the distribution of phospholipids in the plasma membrane (44) as well as the activation of floppases (45), which actively export PS to the outside. Amongst the latter, ATP-binding cassette (ABC) – transporters have been reported to play a role (46). However, the details of PS externalization during apoptosis so far remain elusive. In addition to PS, other 'eat-me' signals have been identified, such as thrombospondin-1, C1q and C3b/bi binding sites and surface structures which are similar to oxidized LDL (oxLDL). The recognition of 'eat-me' signals occurs via specific receptors present on the surface of phagocytes either directly or indirectly via the mediation of soluble bridging proteins. To date several receptors have been identified to bind externalized PS directly, including Tim-1, Tim-4, BAI1 and Stabilin-2 (47-50). The recognition of externalized PS on the apoptotic cell surface by the phagocytic cell can also be mediated by bridging proteins. Arur and co-workers showed that anx A1 can bridge between the phagocyte and externalized PS on the apoptotic cell surface (51-52). So far, the corresponding phagocyte receptor which interacts with anx A1 remains to be identified. Other bridging proteins involved in the recognition of apoptotic cells are MFG-E8 (milk-fat-globule-EGF-factor 8), Gas6 (growth-arrest-specific 6) and $\beta_2$-GPI ($\beta_2$-glycoprotein-I). They can bind to different phagocyte receptors, including the vitronectin receptor $\alpha_V\beta_{3/5}$, the receptor tyrosine kinase Mer and members of the LRP family (53-56). The recognition of thrombospondin-1, C1q and C3b/bi binding sites by the phagocyte receptors CD36/vitronectin and the complement receptors CR3 and CR4 is indirectly mediated by binding of thrombospondin-1 and the complement factors C1q and C3b/bi (57-58). For interaction with oxLDL like binding sites a group of receptors have been identified, which are known as scavenger receptors. SR-A (class A macrophage scavenger receptor), LOX-1 (lectin like oxLDL-receptor 1), CD68, and CD36 (59-60) belong to the scavenger receptor family. The close contact between apoptotic cell and phagocyte initiates the subsequent internalisation and degradation of the apoptotic cell.
In contrast to apoptotic cell clearance recognition of necrotic cells by phagocytes is less well understood. Different groups could show that necrotic cells also externalize PS (61-62) and that they can be recognized through a PS-dependent mechanism, although less efficiently than apoptotic cells (63). Consequently, several macrophage receptor systems, including the thrombospondin-CD36-$\alpha_V\beta_3$ complex and CD14, known to be involved in the uptake of apoptotic cells have been implicated in necrotic cell clearance (64). Furthermore different groups demonstrated an involvement of diverse bridging proteins, such as mannose-binding lectin (MBL), pentraxin-3 and members of the complement protein family, which bind to late apoptotic and necrotic cells (19). Another candidate who

facilitates necrotic cell removal by professional phagocytes is the histidine-rich glycoprotein (HRG), a multidomain plasma protein, which binds selectively to necrotic cells (65-66). Moreover, similarities between pathogen and necrotic cell recognition and phagocytosis are discussed. There are different molecules used by the innate immune system to detect both pathogens and dying cells, in particular plasma membrane-damaged cells (primary or secondary necrotic cells) such as C1q, MBL, CRP, HRG and TLRs (19).
Under physiological conditions secondary necrotic cells occur due to a lack of functional scavenger cells or molecules involved in the removal process. Different bridging proteins are described to bind to apoptotic cells rather late in the cell death process, for example C1q and mannose-binding lectin (MBL) (63). It could be shown that IgG antibodies present in sera of SLE patients are able to opsonize secondary necrotic cell derived material (SNEC). The opsonization of SNEC was dependent on the presence of DNA in SNEC and supported the uptake by blood-borne non-professional phagocytes (23). HMGB-1 and members of the complement pathways are other molecules involved in secondary necrotic cell removal (67-68). The C-reactive protein is a further molecule, which was demonstrated to bind to the surface of secondary necrotic cells but there was no effect on phagocytosis by human monocyte-derived macrophages detectable (69). Therefore, further investigations about recognition of secondary necrotic cells are needed in particular to improve the treatment of chronic inflammatory disease and autoimmunity.

### 2.2.3 Internalization and post-phagocytic response

The first insights into the mechanisms, which after recognition of the cellular corpse initiate their internalization and degradation by the phagocyte, where gained in studies employing the model organism *C. elegans*. In this worm 7 genes play an essential role for phagocytosis by neighboring cells. The deletion of each of these genes leads to an impairment in efficient apoptotic cell clearance and consequently a detectable persistance of the dead cell corpses (70). The products of the CED (cell death-) genes are arranged in two conserved, partially redundant signaling pathways that regulate the engulfment process: 1.) The CED-2 / CED-5 / CED-10 / CED-12 pathway with the mammalian homologues CrkII / Dock180 / ELMO, which activate CED-10 (Rac1) and thus leads to actin-polymerization and initiate the scavenger to flow around the target cell for the final uptake (71-72). Upstream of Dock180 / ELMO leads the binding of BAI1 (present on the surface of the phagocyte) to PS on the apoptotic cell surface to activation of this pathway (49). 2.) The CED-1 / CED-6 / CED-7 pathway. CED-1 is described as transmembrane receptor that has multiple EGF-like domains in its extracellular region; it has high

homology with mammalian multiple EGF-like domains 10 (MEGF10) (73) and may recognize PS on apoptotic cells (74). The signal transduction occurs via CED-6 which binds intracellularly to CED-1, an adaptor protein with homology to mammalian GULP (PTB [phosphotyrosine-binding] domain-containing engulfment adaptor protein) (75-76). CED-7 is discussed to be a homologous to the ABC transporters that actively transport a variety of substances across the plasma membrane, and was originally suggested to be responsible for externalizing the 'eat-me' signal PS on apoptotic cells (73).

Finally, this signaling pathway also leads to activation of CED-10 (Rac1) and thereby to cytoskeletal rearrangement (77). The molecular mechanisms, which manage the internalization and the subsequent degradation of the apoptotic corpses, are not fully understood. Notably, apoptotic cells are taken up by complete engulfment of apoptotic bodies as single entities that form tight fitting phagosomes, without coingestion of the fluid phase. This was similar to the “zipper”- like mechanism of internalization (5). This view is in contrast to several other observations (63).

The last step of silent removal of apoptotic cells is the degradation of the engulfed cellular corpse and in particular the apoptotic cell derived DNA. This was impressively worked out by investigations of Nagata’s group. They could show that DNase II degrades DNA from apoptotic cells (180bp nucleosomal units) in the lysosomes of the macrophages. A lack of DNase II causes the accumulation of 180bp fragmented DNA in macrophages (78), activates the macrophage to produce various proinflammatory cytokines (79) and leads to development of chronic polyarthritis in DNase II knock out mice. In these mice autoantibodies for example anti-CCP (CCP= cyclic citrullinated peptide) antibody or antibodies against double stranded DNA leads to an increased production of proinflammatory cytokines (80). Efficient apoptotic cell removal results in the elimination of the cellular corpses without induction of inflammatory processes and infliction of stress to the organism. This could be assured by a multitude of investigations that have shwon an active repression of inflammatory processes and immune reactions. Thereby, proinflammatory mediators are transcriptionally inhibited, for example IL-12 (81), and / or secretion of anti-inflammatory cytokines is stimulated, such as IL-10 or TGF-β (*transforming growth factor beta*) (82-83).

The mechanisms of internalization and the resulting post-phagocytic response are different for primary necrotic and apoptotic cells. The uptake of necrotic cells occurs by formation of spacious macropinosomes, which is accompanied with cytoplasmatic membrane ruffling of the ingesting macrophage and co-uptake of the surrounding fluid phase (63). The immunological outcome of the post-phagocytic response after engulfment of necrotic cells can depend on the cell type of the necrotic prey as well as the applied

necrosis stimulus (ATP depletion, lysis or heat induced necrosis). In this context, it has been shown that lysed neutrophils stimulate production of macrophage-inflammatory protein 2 (MIP-2), IL-8, TNF-α and IL-10, whereas lysed lymphocytes do not (84-85). Additionally, 'programmed necrotic' (ATP-depleted) cells clearly inhibited TNF-α secretion, whereas heat-killed cells could not inhibit the proinflammatory reaction by macrophages (85). A further molecule, known to be involved in necrotic cell internalization and regulation of the resulting immune response, is the high mobility group box 1 protein (HMGB-1) (86-87). HMGB-1 activates macrophages through TLR2 and TLR4 as it is the case for LPS (88). Other molecules, which can be released by necrotic cells, are heat-shock proteins (89) and large amounts of uric acid (39). Both can have a proinflammatory effect through TLR2 and TLR4 (90-91).

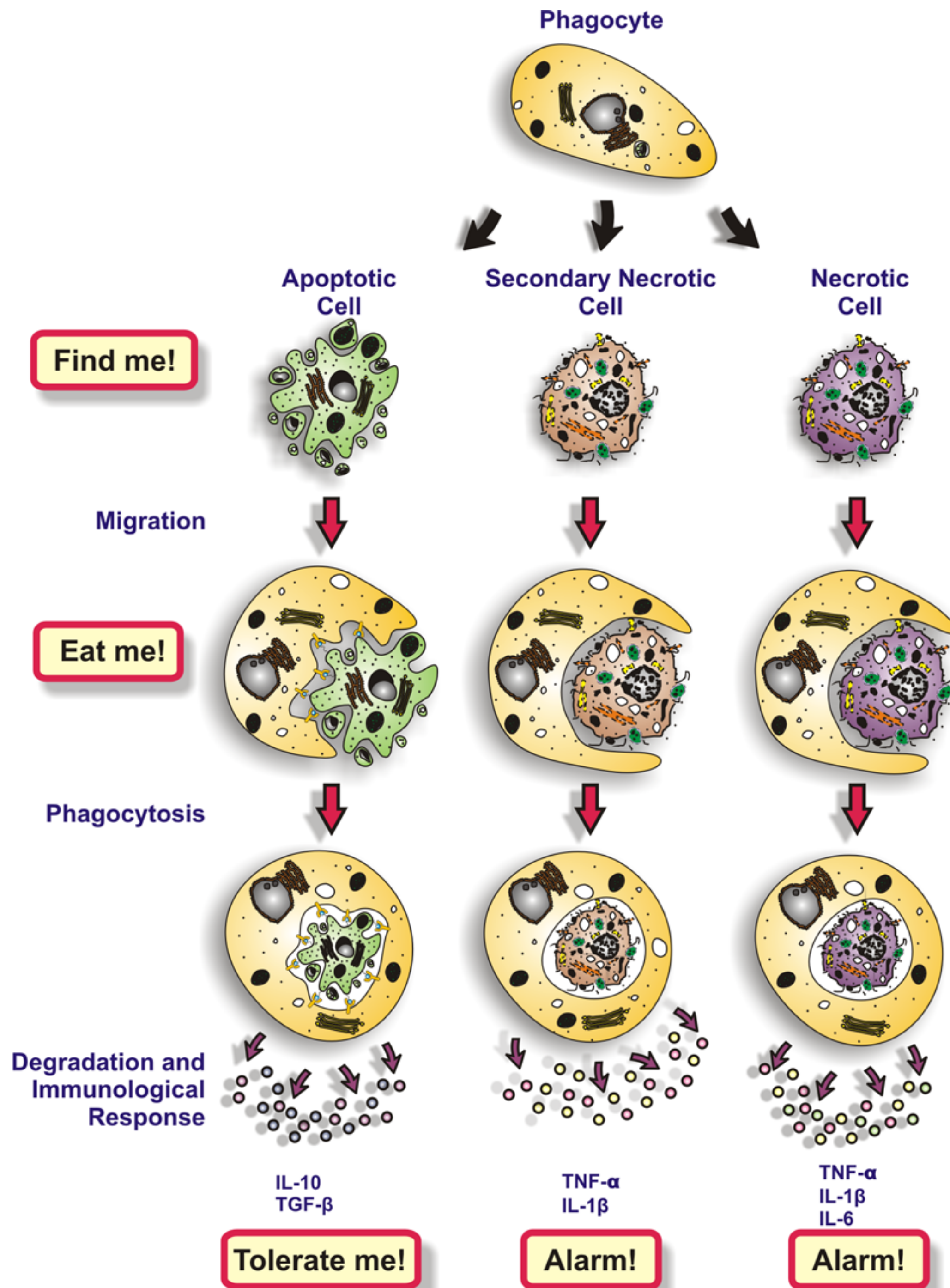


**Figure 2.2: Schematic overview of the three-step process of dying and dead cells elimination:** (1) Apoptotic, secondary and primary necrotic cells liberate 'find-me' signals, which induce attraction of professional phagocytes. (2) Then, the phagocytic synapse forms during close contact between dying cell and scavenger cell. The dying cells present a multitude of 'eat-me' signals on their surface, which are recognized either directly by the phagocyte or indirectly via bridging molecules. Subsequently, the dying or dead prey becomes engulfed by the scavenger. (3) After phagocytosis, the cellular corpses are degraded. At the same time the phagocyte liberates different cytokines to shift the immune reaction to either an anti- or a proinflammatory response depending on the ingested type of prey and the participating scavenger (2).

The mechanisms that govern the internalization of secondary necrotic cells are less well understood. On the one hand, they could be related to those acting with primary necrotic cells, based on a similar morphology of the prey cells with permeable or damaged cytoplasmatic membranes, and on the other hand secondary necrotic cells display similarities to apoptotic cells regarding their cellular content that had been exposed to activated caspases. It could be shown that secondary nectrotic cells injected into the peritoneal cavity of mice are internalized by macrophages (3). Co-culture experiments with secondary necrotic and apoptotic cells revealed that macrophages were less efficient and slower in the ingestion of secondary necrotic cells. Hence, there might be a dependency on the integrity of the plasma membrane (92) and the mechanism of phagocytosis (63). Furthermore, there are controversial reports on the immunological outcome of the internalization of secondary necrotic cells. Some publications report that clearance of secondary necrotic cells by macrophages is not proinflammatory (93-94), whereas a number of publications show that engulfment of secondary necrotic cells may be proinflammatory and immunogenic (95-96). This could be explained by application of early non-ruptured (just permeable cytoplasmatic membrane) or terminal damaged cytoplasmic membranes, the type of prey cells and / or by the use of different types of scavengers (macrophages, dendritic cells or other cell types) (3). Additional work is required to understand in detail the complex interactions between dying cells and phagocytes.

The consequences of delayed or defective apoptotic cell elimination and transition to secondary necrosis in higher organisms could be fatal. Secondary necrotic cells could be the source for autoantigens and by induction of autoantibody production support the development of systemic autoimmune reactions (4, 97-98). In different animal models defects in the attraction of phagocytes and the recognition of apoptotic cells as well as disturbances in their degradation have been shown to have a prolonged impact on the immune system. In mouse models a knock out of the bridging molecule C1q leads to accumulation of apoptotic bodies and development of an autoimmune syndrome that is similar to human systemic lupus erythematosus (99-100). The MFG-E8 knock out mice develop enlarged lymphatic organs and glomerulonephritis caused by aggregation of un-engulfed immune cells and subsequent generation of autoantibodies (101). These results could be confirmed by masking of phosphatidylserine with a dominant negative form of MFG-E8 which also leads to a disturbed phagocytosis of apoptotic cells and generation of autoantibodies (102). Similarly, a defect in the phagocytic receptor-tyrosine kinase Mer supports an accumulation of apoptotic cells and development of a Lupus-like autoimmune syndrome (103). In addition, mice deficient in the LPC-receptor G2A generate a late onset

autoimmune-syndrome (104). Defects in the clearance of apoptotic cells can be caused by extensive apoptosis rates that exceed the present phagocytosis capacity. This could be in conditions when the scavenging capacity is directly impaired by intrinsic defects in the phagocytic function of scavengers or by extrinsic defects in signalling molecules involved in the engulfment process. In this case aggregation of apoptotic cells and shift to secondary necrosis may occur even in situations of normal apoptosis levels (3).

In summary, cells of multicelluar organisms can die in different ways; on the one hand actively by the suicidal death program apoptosis, or passively by necrosis an accidental kind of cell death. The dying and dead cells have to be cleared from the organism and therefore mechanisms for their elimination have to be activated. In the case of apoptotic cells they will be timely cleared by neighbouring cells or attracted phagocytes while the cytoplasmic membrane is still intact thus preventing leakage of intracellular and potentially dangerous molecules. If the clearance process fails, then apoptotic cells undergo secondary necrosis mainly characterized by cytoplasmic membrane damage and cell disintegration. The immunological outcome after ingestion of apoptotic cells is anti-inflammatory whereas engulfment of primary or secondary necrotic cells initiates proinflammatory cytokine secretion and promotes development of autoantibodies. From the current available data it can be suggested that secondary necrosis is implicated in several diseases including autoimmune and neurodegenerative disorders, ischemia and infection. Therefore, the study of post-apoptotic, secondary necrosis is needed to better understand the full potential of this outcome of apoptosis in the promotion of pathologenesis.

## 2.3 Annexin A1

Annexin A1 (also known as annexin I or lipocortin I, Figure 2.3) belongs to the protein family of the annexins and is an amphiphatic protein with a molecular mass of 38 kDa. The annexin family consists of 12 members in humans (A1-A13). They are engaged in different cellular functions for example in membrane reorganization or regulation of intracellular calcium level (105). All annexins have the common ability to bind calcium and phospholipids. Binding is coordinated by four domains (Figure 2.3) which are highly conserved inside the annexin family (106-107). Annexin A1 (anx A1) holds similar to the other annexin family members (excepting anx A6) four of these homologue domains which form a curved protein structure and contain the calcium/phospholipid-binding sites.

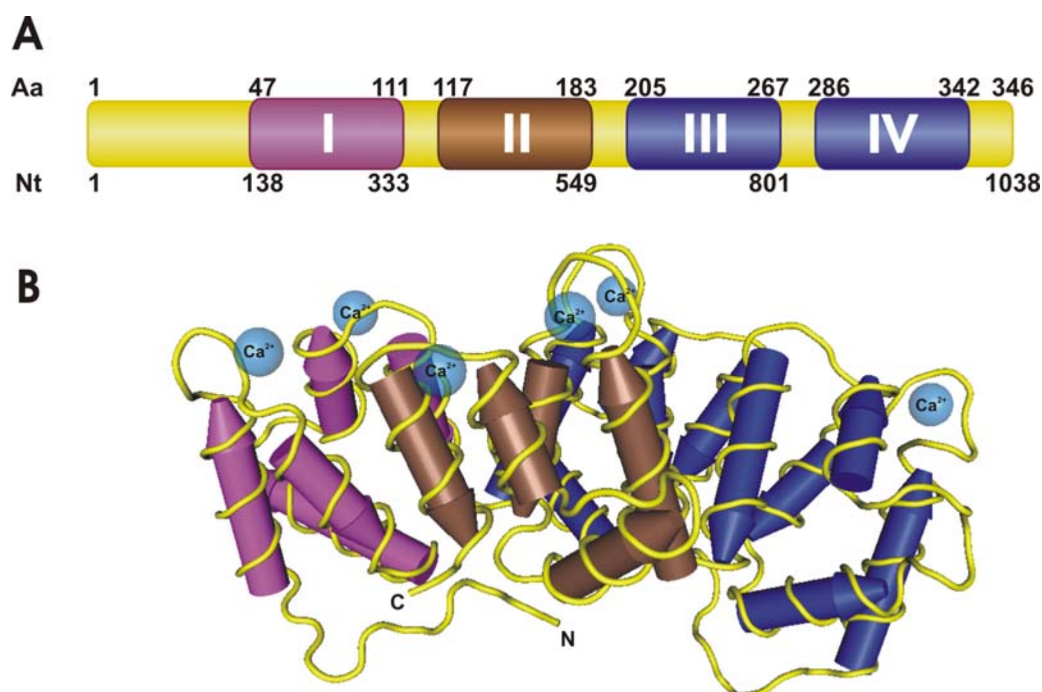


**Figure 2.3: Structure of human annexin A1**
(A) **Schematic illustration of annexin A1:** The illustration shows the monomeric protein (38 kDa) in a linear form. The highlighted regions (I-IV) assign highly conserved calcium- and phospholipid binding motifs, which are known as annexin- boxes. Above and below the schematic are the positions of the annexin-boxes in the amino acid and the nucleotide sequence.
(B) **3-D picture of human annexin A1** (aa33-346, Pub-Med-Nr. 1AIN): Annexin A1 exhibits 20 α-helices and is densely packed in the form of a curved disc. Calcium binding via annexin- boxes occurs exclusively at the convex site. Anx A1 binds per molecule 5 calcium atoms. In the absence of calcium the hydrophobic N-terminal domain is directed to the concave site in the protein core of anx A1. With binding of calcium ions the protein undergoes a conformational change, after which the N-terminus is displaced from the protein core and is freely moveable (thereby not visible in x-ray structure analysis). http://www.ncbi.nlm.nih.gov/Structure/

The different functions of the annexins are determined through the N-terminal domain, which is unique for every family member. Annexin A1 exhibits a 46 amino acids long N-terminus, which inserts into the protein core and upon calcium binding is extruded out by a conformational change (Figure 2.3). This domain can be modified by phosphorylation or proteolytic processing thereby impairing calcium dependent binding to membranes (108). Based on its property to bind to phospholipids – particularly PS – in a calcium dependent manner, anx A1 is able to couple to membranes (109). Moreover, anx A1 has been implicated in different cellular processes, such as cell differentiation and proliferation, membrane trafficking, exo- and endocytosis and organization of the cytoskeleton (110-111). Anx A1 is known as glucocorticoid-inducible protein with various anti-inflammatory functions. Thus, anx A1 inhibits the cytosolic phospholipase $A_2$ ($cPLA_2$) and can therefore repress the release of arachidonic acid and synthesis of proinflammatory mediators (112-113). Furthermore, anx A1 at the surface of monocytes and neutrophils inhibits their

extravasation into inflamed tissue (114-115). Already published data show, that anx A1 is involved in apoptosis (116). Here, anx A1 is externalized by an unknown mechanism to the outer leaflet of the plasma membrane and can be found on PS-rich plaques of dying cells where it functions as a bridging protein and facilitates the phagocytic uptake (51-52).
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 (117). In this context, different groups showed proteolytic processing of anx A1 in the unique N-terminal region by different proteinases, which leads to the release of a peptide, which might be responsible for the anti-inflammatory properties (117-118). Human leukocyte elastase and proteinase 3 are two prominent proteases among others that have been described to be responsible for anx A1 cleavage during neutrophil activation (119-121). Thereby small peptides of different length depending on the cleavage site are generated and secreted. The peptide corresponding to aa 2-26 is the most intensively studied and has been implicated in the induction of monocyte chemotaxis, inhibition of neutrophil extravasation and other anti-inflammatory processes, such as reduction of neutrophil-dependent mouse skin edema, inhibition of neutrophil accumulation in zymosan-induced peritonitis, protection from experimentally induced renal ischemia/reperfusion injury, and amelioration of acute carrageenan-induced inflammation (122-124). Intriguingly, it was also reported that N-terminal anx A1 peptides promote the macrophage-mediated phagocytosis of apoptotic cells (116). Another interesting aspect is shown in a recently published work that describes the detection of anti-anx A1 antibodies present in sera of patients with discoid lupus erythematosus (125).
In conclusion, anx A1 displays an important role in clearance of dying cells as well as in the regulation of the immune system.

# 3. Aims and Results

## 3.1 Cell surface externalization of annexin A1 as a failsafe mechanism preventing inflammatory responses during secondary necrosis

In all multicellular organisms cells die by apoptosis during daily tissue regeneration. Subsequently, they are quickly recognized, internalized and degraded by neighboring cells and professional phagocytes. Non-cleared apoptotic cells otherwise become secondarily necrotic and release intracellular contents into the surroundings. Causes for the accumulation of non-cleared dying and dead cells include intrinsic defects in phagocytic function or massive apoptosis exceeding the available scavenging capacity, for instance in the context of chronic inflammatory diseases or development of autoimmunity. Experimental evidence collected during the last decade suggests that the accumulation of secondary necrotic debris supports the development of diverse autoimmune diseases, such as systemic lupus erythematosus, and chronic inflammatory conditions, such as rheumatoid arthritis. To ensure the timely and efficiently disposal of apoptosing cells a complex network of interactions between the apoptotic cell and the phagocyte has evolved: the so-called phagocytic synapse. Liberated 'find-me' signals, externalized 'eat-me' signals, bridging proteins and the corresponding phagocyte receptors display a multitude of signaling molecules for the removal of apoptotic cells. A prototypical 'eat-me' signal is phosphatidylserine (PS), a phospholipid, that in vital cells is localted at the inner leaflet of the plasma membrane. The translocation of PS from the inner to the outer leaflet of the plasma membrane is a pivotal event in the early phase of apoptosis. Various phagocyte receptors can bind to PS directly or alternatively via bridging proteins. Annexin A1 (anx A1) is one of these bridging proteins. During cell death anx A1 is externalized on the cell surface and can be found on PS-rich plaques. Besides, anx A1 is involved in the regulation of the immune system and has been assigned to an anti-inflammatory role at various control levels.

Disturbances in apoptotic cell clearance and accumulation of secondary necrotic debris have been shown to enhance the onset of autoimmunity and chronic inflammation. Therefore, new therapeutic approaches for treatment of autoimmune diseases could be found by a better understanding of dying cell removal and modulation of inflammation. The main aim of publication 1 was to investigate the role of anx A1 for the still rather unknown engulfment mechanism of secondary necrotic cells regarding the phagocytosis of secondary necrotic cells and the post-phagocytic response of monocytes. It turned out, that anx A1 is strongly externalized on the cell surface after transition from apoptosis to secondary necrosis, and represents a rather late event in comparison to PS exposure.

Moreover, it could be shown that the opsonization of apoptotic cells with soluble recombinant anx A1 strongly facilitates their phagocytic uptake but fails to promote the uptake of secondary necrotic cells, suggesting that the removal of apoptotic and secondary necrotic cells is mediated by distinct pathways. Interestingly, we demonstrated that anx A1 efficiently prevents the secretion of proinflammatory cytokines in macrophages after ingestion of secondary necrotic cells. The results of publication 1 therefore suggest that anx A1 exposure during secondary necrosis provides an important failsafe mechanism counteracting inflammatory responses, even when the timely clearance of apoptotic cells has failed.

## 3.2 Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic 'find-me' signal

In the second publication of this thesis, we wanted to investigate the phenomenon of anx A1 externalization in more detail. Remarkably, we observed that translocation of anx A1 during secondary necrosis coincides with proteolytic cleavage of anx A1. Anx A1 processing was only in anx A1 externalizing cells detectable when these cells convert from apoptosis to secondary necrosis. Furthermore, the anx A1 truncated fragment was only detecable at the secondary necrotic cell surface but not inside the cytoplasm. By administration of different proteinase inhibitors and knock down of protein expression targeting ADAM10 and ADAM17 with specific small interfering RNA transformation during inductiction of secondary necrosis, we could identify the responsible proteinase for anx A1 cleavage as ADAM10.

By employing Edman degration we could locate the cleavage site after $F^7$. Hence, proteolytic processing of anx A1 by ADAM10 generates a small N-terminal peptide that is released from the secondary necrotic cell surface. In the next step we analyzed the consequences of this peptide release in detail. Most interestingly, we could show that the anx A1 N-terminal peptide as well as cell culture supernatants from secondary necrotic anx A1 externalizing cells induced monocyte migration. In contrast, THP-1 monocyte attraction was strongly reduced in cell culture supernatants of secondary necrotic anx A1 silenced cells as well as in ADAM10 silenced cells. Thus, our results show that anx A1 externalization and cleavage provide an important mechanism not only by promoting the removal of apoptotic cells and preventing proinflammatory cytokine production but also by mediating the attraction of monocytes to the site of secondary necrosis.

# 4. Discussion

## 4.1 Cell surface externalization of annexin A1 as a failsafe mechanism preventing inflammatory responses during secondary necrosis

The development of autoimmunity and chronic inflammation is known to be caused by disturbances in apoptotic cell clearance and the concomitant onset of secondary necrosis. A molecularly targeted therapy of autoimmune diseases could aim at the advanced uptake of apoptotic and secondary necrotic cells. In this context, anx A1 seems to be a promising candidate, which belongs to the bridging proteins of the phagocytic synapse, is externalized to the cell surface during cell death and can opsonize apoptotic cells for phagocytosis. In the first part of this thesis, we could show that anx A1 does not only promote the phagocytic uptake of apoptotic cells, but in addition attenuates 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 the prevention chronic inflammation and autoimmunity.

Consistent with previous studies (51-52), which demonstrate the externalization of anx A1 and its role in the phagocytic synapse, we could show that coating of apoptotic cells with soluble recombinant anx A1 also promotes their phagocytosis. Furthermore, we could observe that the core domain of anx A1 alone was sufficient for this activity whereas the N-terminus alone had no enhancing effect on phagocytosis. This might be explained by a bridging of externalized PS on the dying cell with PS on the macrophage surface (126). It was reported by others 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 (116). Our results therefore suggest that different domains of anx A1 may trigger different activities in *cis* or *trans* depending on the target cell. Moreover, we could demonstrate that only anx A1 but not the closely related anx A2 and anx A7 were externalized during cell death. A detailed time course analysis revealed that the externalization of anx A1 was strongly delayed in comparison to PS exposure and predominantly detected in cells that had already lost their membrane integrity. Previous studies 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 (51-52). Thus, our data clearly indicate that anx A1 externalization is an event of secondary necrosis. Though, we observed 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, which could be shown by different

approaches suggesting that distinct mechanisms might be responsible for secondary necrotic cell clearance (2).

A major finding in the first publication of this thesis is, that anx A1 exposure exerts a potent anti-inflammatory action on macrophages following the ingestion of secondary necrotic cells. Anx A1 is known as a crucial mediator of anti-inflammatory glucocorticoid action (117), but its role in postphagocytic macrophage reaction has not been addressed so far. In general, secondary necrotic cells are assumed to exert a proinflammatory effect by directing cytokine expression (3, 127). Our experiments show, that macrophages dose-dependently secrete IL-1 β, TNF, and IL-6 in response to secondary necrotic cell engulfment. But, the amount of proinflammatory cytokines was significantly increased upon ingestion of secondary necrotic cells with silenced anx A1 expression. Suggesting that suppressing of proinflammatory cytokine production is an important function of anx A1, in addition to the already known anti-inflammatory effects. The mechanisms by which anx A1 mediates its anti-inflammatory effects are far from being understood. Externalized anx A1 on the cell surface of dead cells possibly binds to receptors on the phagocyte and thereby influences cytokine production which then influences cytokine production. This notion is corroborated by the observation that purified anx A1 strongly reduced LPS-induced IL-6 production as well as IL-1β and TNF release – though to a lesser extent. The reduction of cytokine release was only observed with full length anx A1 but not with the N-terminal domain or the anx A1 core domain alone. Therefore, different parts of the anx A1 molecule are involved in the control of phagocytosis and cytokine production. The anti-inflammatory activity of anx A1 has been described to inhibit neutrophil extravasation by binding of an N-terminal fragment of anx A1 to members of the formyl-peptide receptor family (128-129). However, in our study the N-terminus alone did not inhibit cytokine production in response to ingestion of secondary necrotic cells; receptors other than formyl-peptide receptors are presumably required for this effect. We can conclude from our data that esposure of anx A1 by dying cells and the concomitant inhibition of postphagocytic IL-1β, IL-6 and TNFsecretion displays a final counteraction to decrease the proinflammatory milieu of secondary necrotic cells.

Therefore, with its anti-inflammatory actions and phagocytosis enhancing effects anx A1 could be an interesting target molecule for futhure therapies of inflammatory disorders associated with defective clearance of dying cells. From our results, it can be concluded that exposure of anx A1 provides an efficient failsafe mechanism counteracting inflammatory responses, even when the timely removal of apoptotic cells has failed.

## 4.2 Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic 'find-me' signal

Defects or delays in the clearance of apoptotic cells lead to initiation of secondary necrosis and are known to promote the onset of chronic inflammation and autoimmunity (4). Acute inflammation, bacterial infections or chemo- and radiotherapy can cause an enormous accumulation of apoptotic cells which exceeds the scavenging capacity (3). During secondary necrosis the plasma membrane becomes permeable and intracellular contents, including cytotoxic metabolic intermediates, hydrolytic enzymes, danger signals, and autoantigens, are released and can stimulate inflammatory and autoimmune reactions.

In the first part of this thesis we could demonstrate that anx A1 is externalized on the secondary necrotic cell suface, that it promotes dying cell uptake, and actively reduces proinflammatory cytokine release by macrophages that have ingested secondary necrotic cells. In the second part of this work, the observation of anx A1 externalization was investigated in more detail. Here, we show that after its translocation to the cell surface anx A1 was proteolytically processed, and identified ADAM10 as the responsible protease, which cleaved anx A1 within its unique N-terminal domain after $F^7$. The subsequently released peptide induced attraction of monocytes. Therefore, anx A1 represents a multi-functional protein at different levels of cell death and cell clearance.

We could show that, anx A1 externalization during secondary necrosis was restricted to certain cell types. Furthermore, we observed that protein extracts from cells, which exhibited anx A1 externalization in the course of secondary necrosis, contained a proteolytically processed, 36 kDa form of anx A1 in addition to the full length protein of 38 kDa. Further kinetic analysis and the observation, that the cleaved 36kDa form was exclusively found on the dying cell surface, support the conclusion that anx A1 is truncated after its translocation to the cell surface although other reports have shown that anx A1 can also be intracellularly processed by caspase-3 during apoptosis (130). Pharmacological inhibition and RNA interference studies identified ADAM10 as the responsible protease that cleaved anx A1 during secondary necrosis. Prominent membrane located subratrates of ADAM10 are for example MICA, CD46, CD95L, and E-cadherin (131-134). ADAM17 is known to be involved in neutrophil apoptosis where it generates a soluble IL-6 receptor fragment which in turn induces monocyte/macrophage recruitment and resolution of inflammation (32). In this study, it was shown that IL-6 receptor shedding occurred in the early onset of apoptosis. But, IL-6R shedding can also be induced by staurosporine and cholesterol depletion, which is dependent on ADAM17 and on ADAM10 (135). However, we could identify the anx A1 N-terminal derived peptide

as a second 'find-me' signal that is generated by ADAM10 activity during cell death. The ADAM10 cleavage site in the anx A1 N-terminus could be sequenced by Edman degradation and was located after $F^7$, which is the first report for this position. Other cleavage sites have been described in the context with other proteases. For instance, during neutrophil activation human leukocyte elastase and proteinase 3 have been shown to process anx A1 at amino acid 26 and between amino acid 29 and 33, respectively (119-121, 136). The most intensivly investigated peptide (aa2-26) has been attributed to induce monocyte chemotaxis, inhibit neutrophil extravasation and other inflammatory processes, such as reduction of neutrophil-dependent mouse skin edema, inhibition of neutrophil accumulation in zymosan-induced peritonitis, protection from experimentally induced renal ischemia/reperfusion injury, and amelioration of acute carrageenan-induced inflammation (122-124). Furthermore, macrophage mediated engulfment of apoptotic cells could be promoted by anx A1 N-terminal peptide (116). The peptide described in the present study (aa2-7) is much shorter in length. However, it strongly induced monocyte chemotaxis. Most importantly, supernatants of secondary necrotic anx A1 or ADAM10 knock-down cells displayed a robust reduction in their chemotactic potential implying that this peptide essentially contributes to monocyte recruitment by secondary necrotic cells.

Members of the formyl peptide receptor family have been identified to mediate effects of anx A1 (aa2-26) peptide (128, 137-138). It has been shown that anx A1 can bind to and activate all three members of the FPR family (118). Remarkably, in the present study only THP-1 monocytes were utilized which express FPR1 and FPR2 but not FPR3 at detectable mRNA levels (Supplemental). A recently published study showed for FPR1 a crucial motif for receptor binding, activation and desensitization which was mapped to anx A1 aa 9-12 (139). This renders FPR1 unlikely to be involved in monocyte migration stimulated by anx A1 aa 2-7, and the responsible receptor remains to be identified in future studies.

Overall, anx A1 represents a protein with various functions at different levels of cell death, dying cell clearance, and anti-inflammation (140). The present study extends this spectrum to a novel function in the context of dying cell removal: Monocyte recruitment by an N-terminal anx A1-derived peptide, that is generated by ADAM10 during secondary necrosis. The multifunctional properties of anx A1 represent attractive targets for novel therapeutic strategies in the context of chronic inflammation and autoimmunity. In this line, local administration of anx A1 or peptides derived from its N-terminus has been reported to alleviate various types of inflammatory reactions (141), and it is tempting to speculate that this might also be a prospective approach for the treatment of inflammatory and autoimmune diseases with known associations to defects in dying cell clearance.

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# 6. Publications

## 6.1 Cell surface externalization of annexin A1 as a failsafe mechanism preventing inflammatory responses during secondary necrosis

This chapter has been published in Journal of Immunology, 2009, 183: 8138 – 8147 by the following authors: Karin E. Blume, Szabolcs Soeroes, Michaela Waibel, Hildegard Keppeler, Sebastian Wesselborg, Martin Herrmann, Klaus Schulze-Osthoff, and Kirsten Lauber.

The author of this thesis performed the majority of experiments (Fig 1, 2B, 3B, 4A, B, E; 5, 6 and supplemental figures) and contributed to write the manuscript. The kinetic analysis of anx A1 externalization, detection of anx A1 externalization after treatment with different stimuli including the measurement of sub G1 nuclei (Fig. 2 A, C and D), and the analysis of externalization of other annexins (Fig. 3A) were done by S.S. The phagocytosis assays with EGTA treated secondary necrotic cells and anx A1 knock down cells (Fig. 4C, D) were performed by M. W.. The analysis of the knock down efficiency of anx A1 on mRNA level, which was not included in the final version of the publication, was done by H. K. which were not included in the final version of the publication. S.W., M.H. and K.S.-O. contributed to write the manuscript. K.L. supervised and designed research and wrote the manuscript.

### 6.1.1 Abstract

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.

### 6.1.2 Introduction

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) on the surface of the apoptotic cell (4). PS can directly be bound by various phagocyte receptors, including BAI1, 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 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.

### 6.1.3 Material 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 1 105 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 Staurosporine 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-anx A1, anti-anx A2, anti-anx 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 nt893: 5’ GGA UUA UGG UUU CCC GUU CUG AAA U3’, anx A1 scramble: 5’GGA UAG GUU CUC CUG CUG UAU UAA U3’.

*Induction of apoptosis and secondary necrosis*

Two x $10^6$ or 1 x $10^5$ cells per well were cultured in 6-well or 96-well plates, respectively. Apoptosis was induced by incubating cells with 2.5 µM staurosporine, 25 µg/ml etoposide, or 100 ng/ml anti-CD95 Ab for the indicated times. Alternatively, cells were UV-irradiated with 10 mJ/cm$^2$ 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 x $10^6$ Jurkat cells were electroporated with 2 $\mu$M of siRNA oligonucleotides in 500 $\mu$l OptiMEM medium (Invitrogen Life Technologies) by a single pulse (800 $\mu$F, 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 $CaCl_2$, 1% heat-inactivated human serum and 0.1% NaN3) supplemented with 0.5–2 $\mu$g/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 $\mu$g/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 x $10^5$ cells per well were washed in staining buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$) and incubated with 0.2 nmol of the different Cy2-labeled anx A1 mutants diluted in 100 $\mu$l staining buffer supplemented with 2 $\mu$g/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 $\mu$g/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 1–46, aa 1–346, 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 (Profos). 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- labeling kit (GE Healthcare) according to the manufacturer's instructions. In brief, 1 mg of recombinant protein was incubated with the reactive dye 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 x $10^5$ 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. Cellfree supernatants were collected and cytokine concentrations (IL-1β, TNF, 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 mJ/cm2, and incubated for the indicated times. Subsequently, apoptotic or postapoptotic cells were added to 1 x $10^5$ 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, $2.5 \times 10^6$ prey cells were pretreated for 30 min at 4°C with 1 nmol of the indicated proteins in 500 µl TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) supplemented with 5 mM $CaCl_2$. 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 $CaCl_2$ before incubation with macrophages.

*Adhesion assay*

Prey cells were stained with PKH67 (Sigma-Aldrich), UV-irradiated with 10 mJ/cm2 and incubated for 12 h. In brief, $2.5 \times 10^6$ prey cells were pretreated with 1 nmol of the indicated proteins in 500 µl TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) supplemented with 5 mM $CaCl_2$ 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 $5 \times 10^4$ PMA-differentiated THP-1 macrophages (ratio 4:1) in 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.

### 6.1.4 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. 1A). 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. 1A). 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).5 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. 1A 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 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.

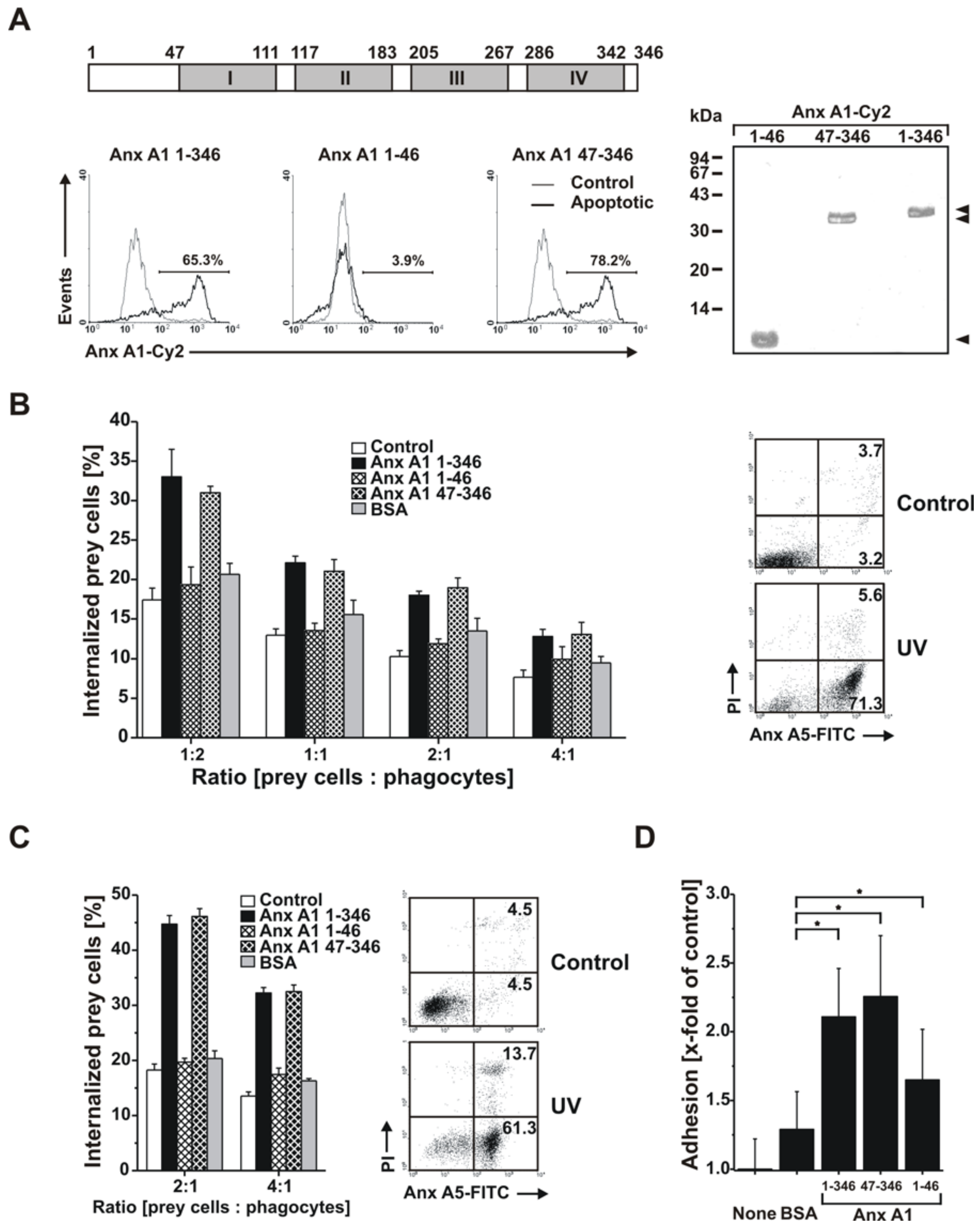


**Figure 1**: **Recombinant soluble anx A1 promotes the phagocytosis of apoptotic cells.** A, Apoptotic Jurkat cells bind Cy2-labeled anx A1 and an anx A1 core fragment. *Upper left*, Domain structure of anx A1. Arabic numbers depict the amino acid position and annexin repeats are numbered I-IV. *Lower 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, stimulated to undergo apoptosis by UV-

irradiation (10 mJ/cm$^2$), incubated for 4 h, and coated with different forms of anx A1 or BSA (1 nmol purified protein per 2.5 x $10^6$ cells). Subsequently, cells were added at the indicated ratios to 1 x $10^5$ PKH67-labeled, PMA-differentiated THP-1 macrophages per well and incubated for 2 h. 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 for apoptosis induction by UV irradiation and further incubation for 12 h. Subsequently, cells were coated with different forms of anx A1 or BSA and added at the indicated ratios to PKH67-labeled THP-1 macrophages as described in B, The percentage of internalized prey cells 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 x $10^6$ cells) as described in C. Subsequently, cells were added at a 4:1 ratio to 5 x $10^4$ PMA-differentiated THP-1 macrophages per well and incubated for 30 min. Bound prey cells were detached in EDTA-supplemented PBS, lysed, and quantified by fluorometry. Adhesion was normalized on protein-free control samples and is given as mean + SD of octaplicates. $p$ values were calculated in comparison to BSA-containing samples by unpaired heteroscedastic Student's t test analysis. *, $p < 0.05$.

*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 analyzed 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).

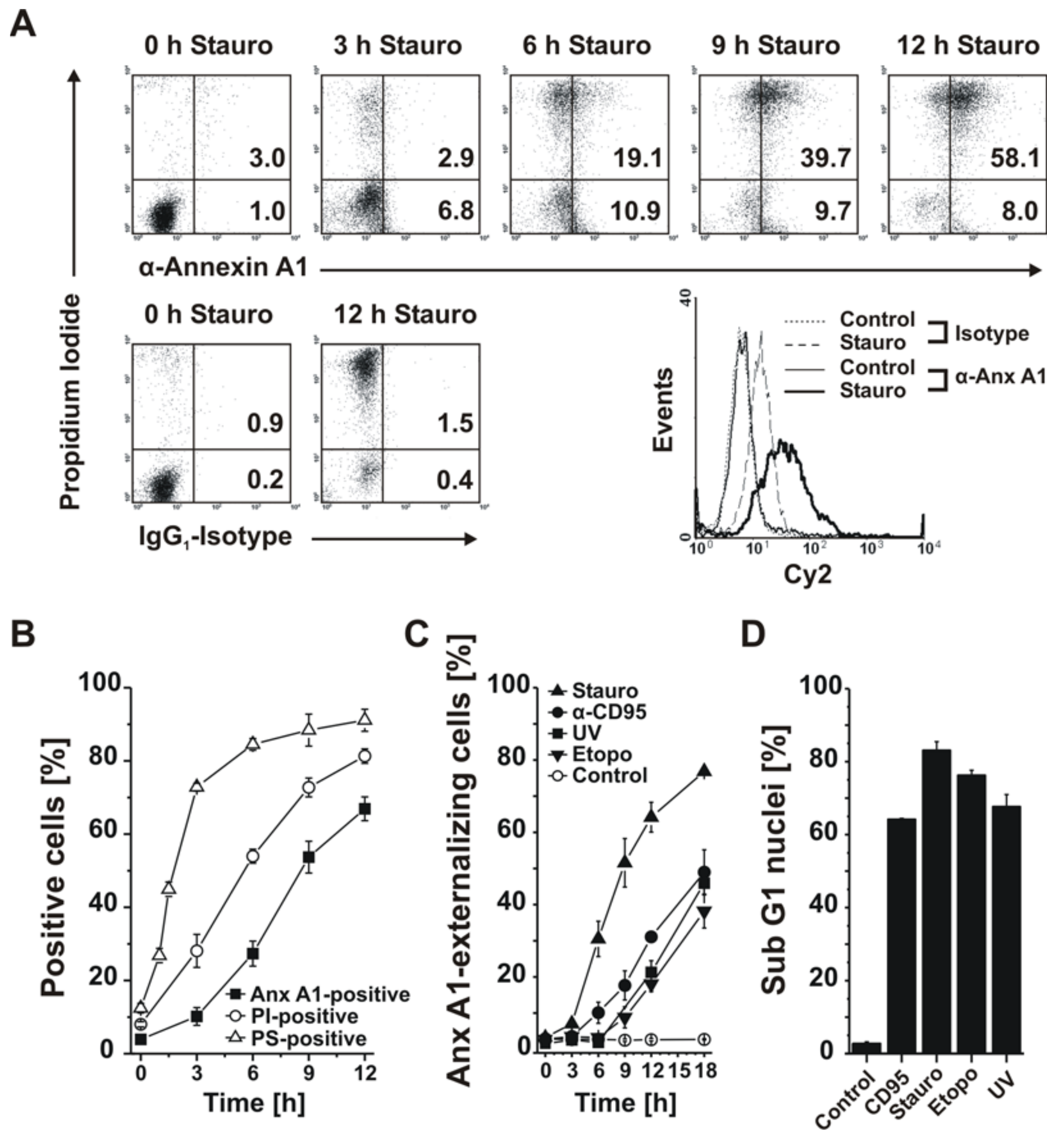


**Figure 2: Time course of anx A1 externalization during apoptosis and secondary necrosis.** A, The majority of anx A1 externalizing cells is secondary necrotic. Jurkat cells were stimulated with 2.5 *µ*M staurosporine (Stauro) for the indicated times to undergo apoptosis followed by secondary necrosis. Subsequently, cells were stained with anti-anx A1/anti-mouse-IgG-Cy2 and PI and analyzed by flow cytometry. Mouse IgG1 served as isotype control. Representative dotplots of triplicates are shown. The histogram displays anx A1 surface expression 12 h after incubation with or without staurosporine. B, Exposure of phosphatidylserine and loss of plasma membrane integrity precede anx A1 externalization. Jurkat cells were left untreated or stimulated as in A. Cell surface exposure of phosphatidylserine (PS) was detected by anx A5-FITC staining. Anx A1 externalization and PI exclusion were measured as in A. The percentages of PS-positive, anx A1-positive and PI-positive cells (mean values ± SDs of triplicates) are shown. C, Different cell death stimuli induce anx A1 externalization with different kinetics. Jurkat cells were left untreated or stimulated with 2.5 *µ*M staurosporine (Stauro), 100 ng/ml anti-CD95 agonistic Ab (α-CD95), 25 *µ*g/ml etoposide (Etopo), or 10 mJ/cm$^2$ UV irradiation (UV) for the indicated times. Anx A1 externalization was measured as in A. Mean values ± SDs of triplicates are given. D, Induction of DNA fragmentation by different cell death stimuli, Jurkat cells were stimulated to undergo apoptosis followed by secondary necrosis as in C. After 24 h cell death was measured by flow cytometry and calculated as percentage of subdiploid nuclei (mean values ± SDs of triplicates).

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 actinassociated 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 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).

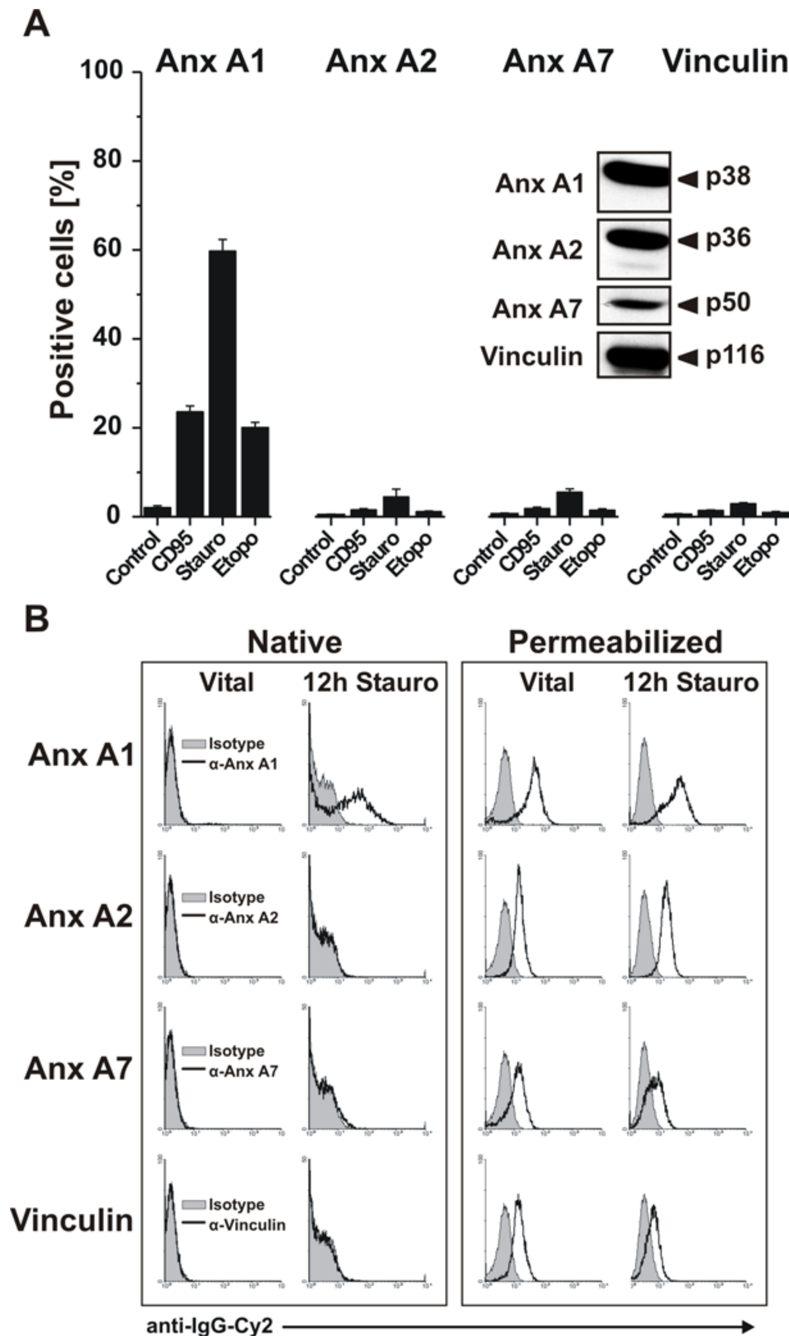


**Figure 3: Externalization during cell death is not a general feature of the annexin family.** A, Different cell death stimuli induce externalization of anx A1 but not anx A2 or anx A7. Jurkat cells were stimulated as in Fig. 2C for 12 h and stained with anti-anx A1, anti-anx A2, anti-anx A7, or anti-vinculin and anti-mouse-IgG-Cy2.The percentages of cells with positive staining signal are shown as mean values + SDs from triplicates. The inlay shows an immunoblot analysis of anx A1, anx A2, anx A7, and vinculin expression in Jurkat cells. B, Staining of different annexins and vinculin in native and permeabilized cells. Jurkat cells were stimulated as in A and either stained natively *(left)* or following fixation/permeabilization *(right)*.

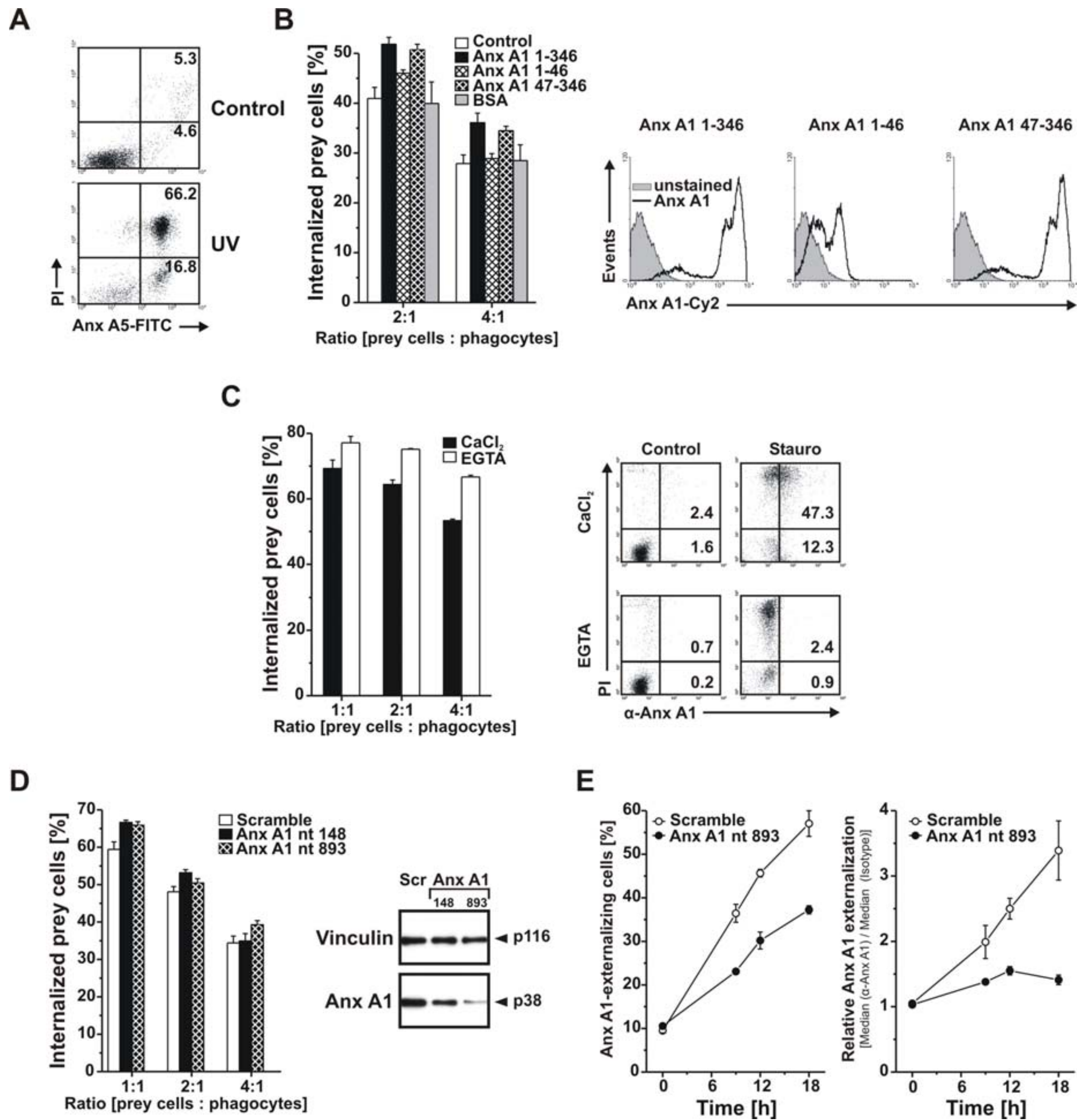


**Figure 4: Phagocytosis of secondary necrotic cells occurs independently of anx A1.** A, Induction of secondary necrosis: Jurkat cells were stimulated to undergo secondary necrosis by UV-irradiation (10 mJ/cm$^2$). After 18 h, PS externalization and plasma membrane integrity were measured. Representative 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 x 10$^6$ cells) as in Fig. 1B and fed in different ratios to 1 x 10$^5$ 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/cm$^2$). Fourteen hours after irradiation, cells were washed in TBS with 5 mM $CaCl_2$ 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 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 + SDs from quadruplicate experiments. *Right*, Immunoblot analysis of anx A1-silenced or scramble control Jurkat cells confirmed the efficient down-regulation of anx A1 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.

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, dosedependently 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-1 β, 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.

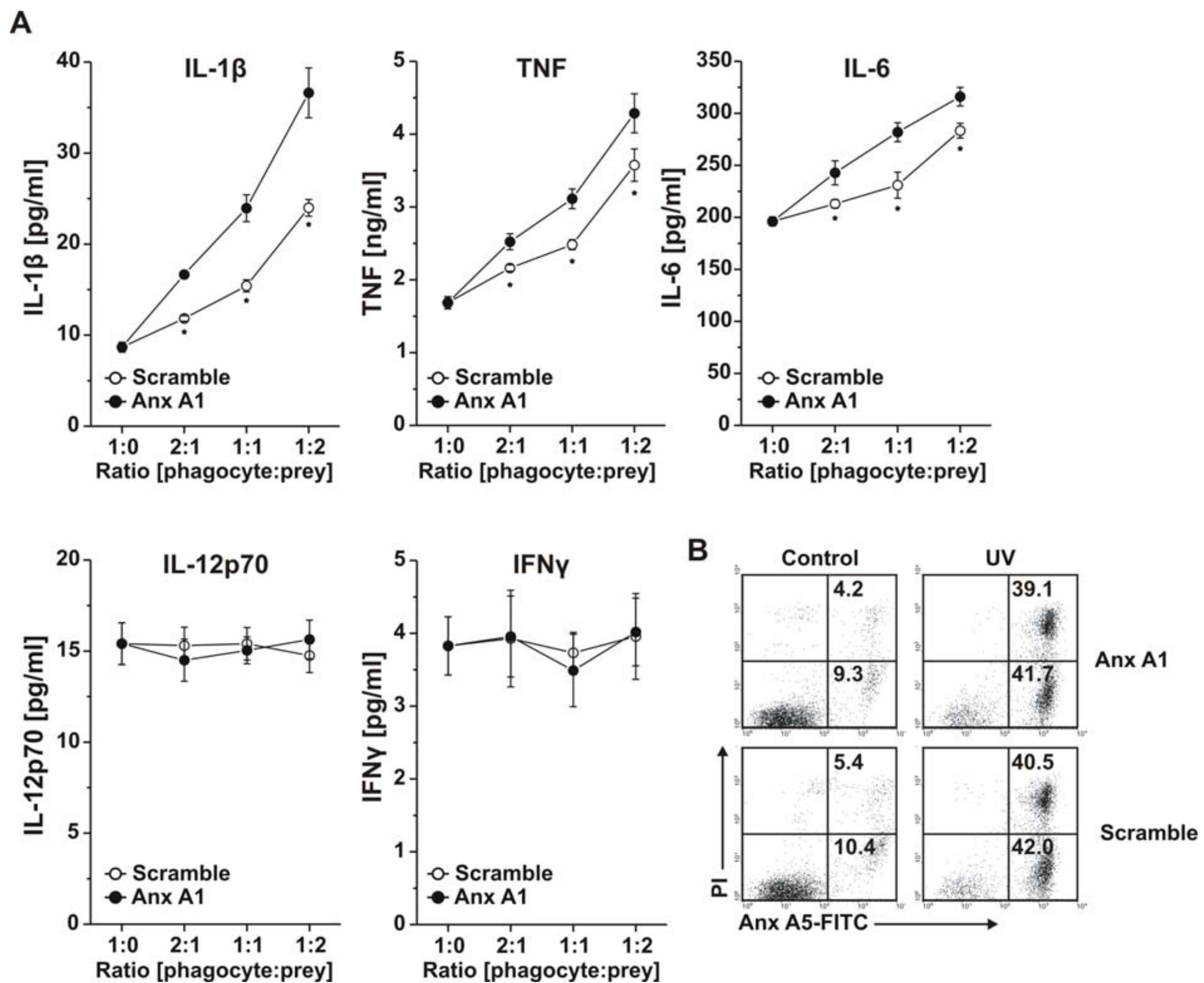


**Figure 5: Secondary necrotic anx A1 knock-down cells exhibit an enhanced proinflammatory potential.** A, Proinflammatory cytokine production by macrophages after ingestion of secondary necrotic anx A1 knock-down or scramble control Jurkat cells. PMA-differentiated THP-1 macrophages (1 x $10^5$ cells/well in 24-well plates) were fed at different ratios with secondary necrotic anx A1-silenced (oligonucleotide anx A1 nt893) or scramble control Jurkat cells (18 h post 10 mJ/$cm^2$ UV) for 2 h. Subsequently, non-engulfed prey cells were washed away and macrophages were further incubated with 100 ng/ml LPS for 24 h. Cytokine levels in supernatants were determined by ELISA as described in *Materials and Methods*. Mean values ± SD of octaplicates are given. *p* values were calculated between pairs of equal phagocyte : prey ratio by unpaired heteroscedastic Student's t test analysis. *, $p < 0.01$. B, Apoptosis and secondary necrosis are not affected by anx A1 knock-down. PS externalization and PI exclusion were analyzed in Jurkat prey cells used in A by flow cytometry as described in Fig. 4A. Representative dot plots of triplicates are shown.

*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 fulllength 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.

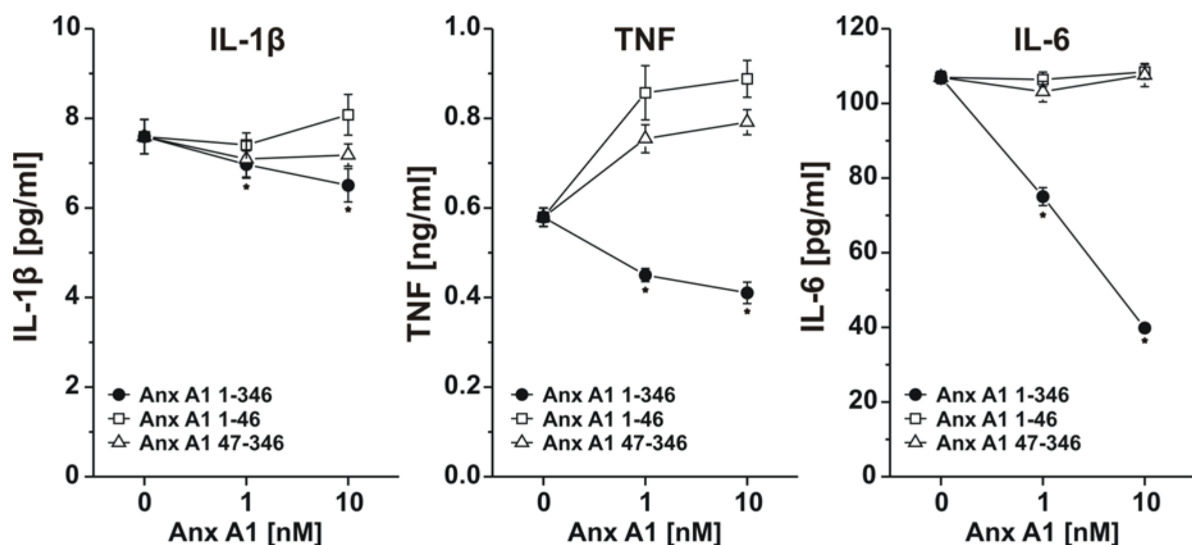


**Figure 6: Exogenous anx A1 inhibits proinflammatory cytokine production by macrophages.** PMA-differentiated THP-1 macrophages (1 x $10^5$ cells/well) were stimulated with 100 ng/ml LPS for 24 h in the presence or absence of different concentrations of purified anx A1 constructs. Cytokine levels in supernatants were determined by ELISA as described in *Materials and Methods*. Mean values ± SD of octaplicates are given. *p* values were calculated between the incubation with full-length anx A1 and the incubation without anx A1 by unpaired heteroscedastic Student's t test analysis. *, $p < 0.01$.

### 6.1.5 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 erythematosus 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 action 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 cytokine 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 proinflammatory 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 effects 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 phagocytosis 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 apoptotic 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. Interestingly, 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 phagocytosis 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 production 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 secondary necrotic cells, exposure of anx A1 provides an efficient failsafe mechanism counteracting inflammatory responses, even when the timely removal of apoptotic cells has failed.

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### 6.1.7 Supplemental

**Supplemental Methods**

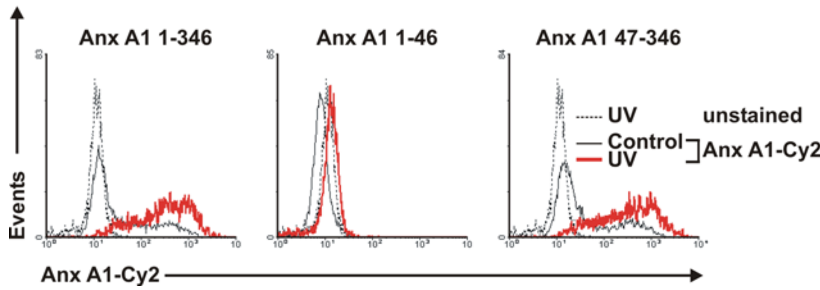
*Preparation of primary human PHA-stimulated lymphoblasts, monocytes and neutrophils*

Human PBMCs and neutrophils were prepared from heparinized blood by double-Ficoll gradient purification (Histopaque 1.119 g/ml, Sigma and Ficoll-Paque 1.077 g/ml, GE Healthcare). Primary human monocytes were positively selected from PBMCs with anti-CD14 magnetic beads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's recommendation. PHA-stimulated lymphoblasts were prepared by cultivating monocyte-depleted human PBMCs for 2 days in RPMI-1640 supplemented with 10% heatinactivated fetal calf serum, 100 units of penicillin/ml, 0.1 mg streptomycin/ml, 10 mM HEPES and 0.5 µg/ml PHA (Biochrom, Berlin, Germany) and 5 further days in PHA-free medium.

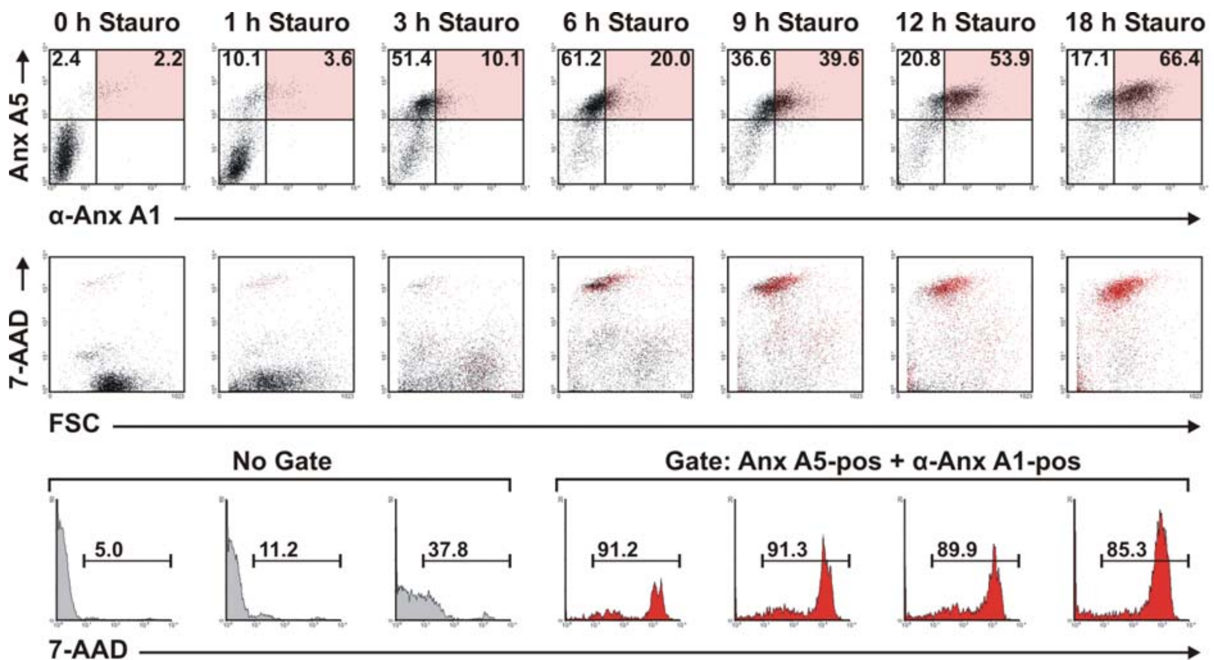
*Flow cytometric triple staining for PS exposure, membrane permeabilization and anx A1 externalization*

Staining with the GFP-certified apoptosis/necrosis detection kit (Enzo Life Sciences, Loerrach, Germany) was performed according to the manufacturer's instructions. Anti-anx A1 staining was combined with the procedure by pre-incubating the cells with anti-anx A1 for 30 min and subsequently adding the secondary anti-mouse-IgG-Cy2 antibody to the anx A5-Cy3/7-AAD staining mixture.

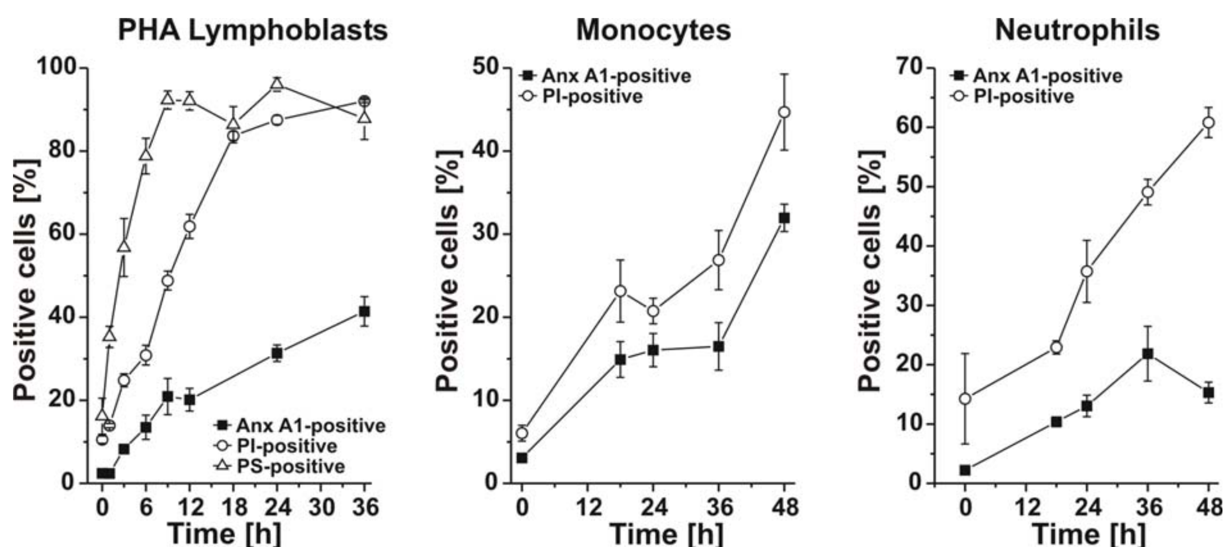
## Supplemental Figures




**Supplemental Figure 1: Apoptotic cells bind Cy2-labeled anx A1 and an anx A1 core fragment.** THP-1 cells were left untreated or UV-irradiated with 10 mJ/cm$^2$. After 12 h of incubation 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).




**Supplemental Figure 2: Time course of phosphatidylserine exposition, membrane permeabilization and anx A1 externalization during apoptosis and secondary necrosis.** Jurkat cells were stimulated with 2.5 μM staurosporine (Stauro) for the indicated times in order to undergo apoptosis followed by secondary necrosis. Subsequently, cells were stained with anti-anx A1/anti-mouse-IgG-Cy2, anx A5-Cy3 and 7-AAD and analyzed by flow cytometry. *Upper panel:* Dotplots showing anti-anx A1 vs. anx A5 staining. *Middle panel:* Dotplots showing FSC vs. 7-AAD. Cells positive for anti-anx A1 and anx A5 staining (upper right quadrant in upper panel) are displayed in red. *Lower panel:* Histograms of 7-AAD fluorescence. The first 3 histograms display the whole population analyzed, whereas the last 4 histograms are gated on the population positive for anti-anx A1 and anx A5 (upper right quadrant in upper panel). Representative plots of triplicates are shown.




**Supplemental Figure 3: Exposure of phosphatidylserine and loss of plasma membrane integrity precede anx A1 externalization in primary human PHA-stimulated lymphoblasts, monocytes and neutrophils.** Primary human PHA-stimulated lymphoblasts, monocytes and neutrophils were stimulated with 2.5 µM staurosporine to undergo apoptosis and secondary necrosis. Cell surface exposure of phosphatidylserine (PS) was detected by anx A5-FITC staining. Anx A1 externalization and PI exclusion were measured as in Fig. 2A. The percentages of PSpositive, anx A1-positive and PI-positive cells (mean values ± standard deviations of triplicates) are shown.

## 6.2 Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic 'find-me' signal

This chapter has been submitted for publication in Journal of Immunology by the following authors: Karin E. Blume, Szabolcs Soeroes, Hildegard Keppeler, Stefan Stevanovic, Dorothee Kretschmer, Maren Rautenberg, Sebastian Wesselborg and Kirsten Lauber.

The author of this thesis designed and performed most of the experiments (Fig 2B, 3A-E, G, 4B-D, 5, 6 and supplemental figures) and contributed to write the manuscript. The detection of anx A1 externalization in different cell lines during secondary necrosis and anx A1 expression in all utilized cell types (Fig. 1) was done by S.S. and Edman degradation analysis (Fig. 4E) was performed by S.St.. The analysis of the knock down efficiency of ADAM10 on mRNA level was done by H. K. (Fig 3F). D.K. and M.R. performed in vivo experiments which were not included in the final version of the manuscript. S.W. contributed to write the manuscript. K.L. supervised and designed experiments and wrote the manuscript.

### 6.2.1 Abstract

Annexin A1 is an intracellular calcium/phospholipid-binding protein that is involved in membrane organization and the regulation of the immune system. It has been attributed

an anti-inflammatory role at various control levels, and recently we could show that annexin A1 externalization during secondary necrosis provides an important failsafe mechanism counteracting inflammatory responses, when the timely clearance of apoptotic cells has failed. As such, annexin A1 promotes the engulfment of dying cells and dampens the post-phagocytic production of proinflammatory cytokines. In our present follow-up study, we report that exposure of annexin A1 during secondary necrosis coincided with proteolytic processing within its unique N-terminal domain by 'a disintegrin and metalloprotease 10' (ADAM10). Most importantly, we demonstrate that the released peptide as well as culture supernatants of secondary necrotic, annexin A1 externalizing cells induced chemoattraction of monocytes, which was clearly reduced in annexin A1- or ADAM10-knock-down cells. Thus, altogether our findings indicate that annexin A1 externalization and its proteolytic processing into a chemotactic peptide represent final events during apoptosis, which after the transition to secondary necrosis contribute to the recruitment of monocytes and the prevention of inflammation.

### 6.2.2 Introduction

Annexin A1 (anx A12, also known as annexin I or lipocortin I) is a glucocorticoid-inducible member of the annexin superfamily, which comprises 13 members that have specific biological functions in membrane reorganization and the regulation of intracellular calcium levels (1, 2). All annexins have the common ability to bind calcium and phospholipids coordinated by four or more highly conserved domains, the so-called annexin boxes (3, 4). The different functions of the annexins are determined by their unique N-terminal domains. Anx A1 exhibits a 46 amino acid long N-terminus that in the absence of calcium is inserted into the protein core and upon calcium binding is extruded by a conformational change. This domain is known to undergo posttranslational modifications, including phosphorylation, which fine-tune calcium-dependent binding of anx A1 to membranes (5). Additionally, peptides derived from the anx A1 N-terminus can be generated by different proteases and exhibit anti-inflammatory properties (6, 7). Based on its capability to bind phospholipids, in particular phosphatidylserine (PS), anx A1 can couple membranes and has been implicated in different cellular processes, such as differentiation and proliferation, membrane trafficking, exo- and endocytosis, and organization of the cytoskeleton (8, 9). Extracellularly, bound to the surface of monocytes and neutrophils anx A1 has been described to inhibit their extravasation into inflamed tissues (10, 11). Anx A1 is also known to be involved in apoptosis. Thus, it has been reported to be externalized by so far unknown mechanisms and to bind to PS-rich plaques on the surface of dying cells, where it functions as a bridging protein facilitating their phagocytic uptake (12, 13). Our

previous studies have shown that externalization of anx A1 is a rather late apoptotic event occurring after the transition from apoptosis to secondary necrosis. In this context, externalized anx A1 potently inhibits the release of proinflammatory cytokines by macrophages that have engulfed secondary necrotic cells (14).

Secondary, post-apoptotic necrosis occurs if the phagocytic removal of apoptotic cells fails, for instance when the phagocytic process is directly impaired or when massive apoptosis overwhelms the available scavenging capacity. Such situations have been described in the context of acute inflammation associated with massive neutrophil accumulation, ischemia, drug-induced hepatitis, or bacterial infections (15). During the transition from apoptosis to secondary necrosis the integrity of the plasma membrane becomes compromised leading to the leakage of potentially cytotoxic and antigenic intracellular contents into the surrounding tissue. This in turn can promote and propagate the onset of inflammatory and autoimmune responses. Consequently, several autoimmune diseases, such as systemic lupus erythematosus, and chronic inflammatory conditions, such as rheumatoid arthritis, have been linked to a deficient and/or delayed clearance of apoptotic cells (15, 16).

In the present study, we have investigated the phenomenon of anx A1 externalization during secondary necrosis in greater depth. We observed that anx A1 externalization coincided with proteolytic cleavage of anx A1 within its unique N-terminal domain. Pharmacological inhibition and RNA interference experiments identified ADAM10 as the responsible protease in this scenario. Mapping of the cleavage site by Edman degradation revealed a small peptide comprising the N-terminal 7 amino acids that was generated and presumably released from the secondary necrotic cell surface. Most interestingly, this peptide as well as cell culture supernatants from secondary necrotic anx A1 externalizing cells induced monocyte migration. In contrast, culture supernatants of secondary necrotic anx A1-silenced as well as ADAM10-silenced cells displayed a profound reduction in their monocyte-attracting potential. Thus, our results show that externalized anx A1 exerts multiple functions on diverse levels during secondary necrosis not only by promoting the phagocytic removal of dying cells and preventing proinflammatory cytokine production but also by recruiting monocytes to the sites of secondary necrosis.

### 6.2.3 Material and Methods

*Cells and cell lines*

Jurkat, THP-1, MOLT-4, HuT78, HL60 (all from ATCC) and U937 cells (a gift from Ralph Hass) were cultured in RPMI1640 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, Karlsruhe, Germany). PHA-stimulated human T lymphoblasts (PHA blasts) were generated as described previously (14). Cells were grown at 37°C in a 5% CO2 atmosphere and maintained in log phase.

*Antibodies, small interfering RNA (siRNA) oligonucleotides, and other reagents*

Staurosporine was purchased from Roche Molecular Biochemicals (Penzberg, Germany). The following mAbs were used for flow cytometry and immunoblot analyses: anti-anx A1 (from BD Biosciences, Heidelberg, Germany), anti-vinculin (Sigma-Aldrich, Taufkirchen, Germany), anti-PARP (Axxora, Lörrach, Germnay), anti-ADAM10 and anti-ADAM17 conjugated to FITC or PE (R&D Systems, Heidelberg, Germany). In Fig. 4C a polyclonal anti-anx A1 antibody (Invitrogen) was employed. Chemically modified siRNA oligonucleotides (stealth RNAi) were purchased from Invitrogen. The following sequences were used: anx A1 nt893: 5'-GGA UUA UGG UUU CCC GUU CUG AAA U-3', anx A1 scramble: 5'-GGA UAG GUU CUC CUG CUG UAU UAA U-3', ADAM10 nt715: 5'-CGA GAA GCU GUG AUU GCC CAG AUA U-3', ADAM10 nt2064: 5'-GAU CAU GCU AAU GGC UGG AUU UAU U-3', ADAM10 scramble: 5'-CGA UCG AAG UGC GUU GAC CAA GUA U-3', ADAM17 nt90: 5'-GAG ACU CGA GAA GCU UGA UUC UUU G-3', ADAM17 nt969: 5'-UGA UAU AGC UGA GGA AGC AUC UAA A-3', ADAM17 scramble: 5'-GAG AGC UGA AGG UUC CUU AUC AUU G-3'. Different protease inhibitors were used to characterize the protease responsible for anx A1 cleavage: Elastase-inhibitor (Bachem, Weil am Rhein, Germany), pefabloc SC and protease-inhibitor cocktail (Roche), aprotinin and o-phenanthroline (Sigma-Aldrich), GM6001 and TAPI-2 (Merck Chemicals, Darmstadt, Germany), GI254023X and GW280264X (both from Andreas Ludwig, RWTH Aachen, Germany), and zVAD-fmk (Bachem). The N-terminal anx A1 peptide (aa 2-7, AMVSEF) and the corresponding reverse sequence (aa 7-2, FESVMA) were synthesized in an automated peptide synthesizer 433A (Applied Biosystems, Darmstadt, Germany) following the Fmoc/tBu strategy. Synthesis products were analyzed by HPLC (Younglin Acme 9000, Younglin, Korea) and ESI-TOF mass spectrometry (Q-ToF, Waters, Eschborn, Germany).

*Induction of apoptosis and secondary necrosis*

1 x 106 or 1 x 105 cells per well were cultured in 24-well or 96-well plates, respectively. Apoptosis was induced by addition of 2.5 μM staurosporine, or cells were UV irradiated with 10 mJ/cm2 in the UV Stratalinker 2400 (Stratagene) as described previously (17) and incubated for the indicated times. Protease inhibitors were added 5 h after stimulation with staurosporine or UV irradiation.

*Production of culture supernatants of viable and secondary necrotic cells*

2 x 106 cells per ml were UV irradiated with 10 mJ/cm2 and incubated in serum-free medium for the indicated times. Supernatants were collected by centrifugation at 4,000 x g for 10 min and stored at -70 °C.

*Flow cytometry*

Flow cytometry was performed with a FACSCalibur (BD Biosciences) and CellQuest analysis software. Externalization of PS was measured by staining with annexin A5-FITC/PI (Roche) according to the manufacturer's instructions. Cells positive for annexin A5-FITC but negative for PI staining were considered apoptotic, whereas cells double-positive for annexin A5-FITC and PI staining were considered secondary necrotic (14). DNA fragmentation as a marker of apoptosis and secondary necrosis was determined by the percentage of sub G1 nuclei, and anx A1 externalization was assessed by indirect FACS immunostaining as described previously (14, 18). For surface staining of ADAM10 and ADAM17 on native cells, cells were suspended in ice-cold staining buffer (RPMI1640 medium plus 2% heat-inactivated human serum) supplemented with FITC- or PE-conjugated mAb and incubated for 30 min on ice. After two washing steps in staining buffer cells were resuspended in staining buffer and analyzed by flow cytometry. For staining of permeabilized cells (Supplemental Fig. 3), the Cytofix/Cytoperm kit (BD Biosciences) was used according to the manufacturer's recommendations.

*SDS-PAGE, silver staining and Westernblot analysis*

SDS-PAGE and Westernblot analysis were performed as described previously (19) with mAbs against anx A1 (from BD Biosciences), PARP (Axxora) and vinculin (Sigma-Aldrich). Silver staining of SDS-PA gels was performed as described (20).

*Electroporation with siRNA oligonucleotides*

Electroporation with siRNA oligonucleotides was performed 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 x 106 Jurkat cells were electroporated with 2 µM of siRNA oligonucleotides in 500 µl OptiMEM medium (Invitrogen) 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.

*qRT-PCR analysis*

The detection of ADAM10 and ADAM17 mRNA levels was performed by qRT-PCR analysis with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and qPCR Maxima Mastermix (Fermentas). The following probes and primers (Sigma-Aldrich) were used: ADAM10 forward, 5'-CGG AAC ACG AGA AGC TGT GAT T-3'; ADAM10 reverse, 5'-TTA CGG ATT CCG GAG AAG TCT GT-3'; ADAM17 forward, 5'-CGA GTA CAG GAT GTA ATT GAA CGA TTT-3'; ADAM17 reverse, 5'-CCA ACG ATG TTG TCT GCT AAA AAC T-3'; ALAS-1 forward, 5'-TCC ACT GCA GCA GTA CAC TAC CA-3'; ALAS-1 reverse, 5'-ACG GAA GCT GTG TGC CAT CT-3'; ALAS-1 probe VIC-5'-AAA GAA ACC CCT CCG GCC AGT GAG AA-3'-TAMRA. Total RNA from 2 x 106 cells was extracted with the Nucleo-Spin® RNA II kit (Macherey & Nagel, Dueren, Germany). 1 µg of RNA was reverse transcribed with 200 units of RevertAid reverse transcriptase in the presence 50 µM random hexamers, 400 µM dNTPs, and 1.6 units/µl RiboLock (all from Fermentas). 40-80 ng of the resulting cDNA were applied to the following qRT-PCR analyses (20 µl final volume) with 200 nM primers and 100 nM probe, or 300 nM primers in the case of SYBR Green based detection. For the study of siRNA knockdown effects, relative quantification was performed employing the standard curve method. The results were normalized on the reference gene ALAS-1, and the cell population transfected with the scramble control oligonucleotide was used as calibrator. All experiments were performed in duplicates and are presented as mean values.

*Transmigration assay*

Transmigration assays were carried out with slight modifications as described previously (21). Briefly, 1 x 105 calcein-labeled THP-1 cells per well were used in a final volume of 75 µl. 300 µl of cell culture supernatants or chemokines dissolved in serum-free RPMI 1640 medium were placed into the lower chamber of 5 µm pore Multiscreen-MIC chemotaxis chambers (Millipore Corp., Billerica, MA, USA). The filter was adjusted, the stained cell suspension was added on top, and the assay was incubated for 120 min at 37°C. Subsequently, the transmigrated cells were collected by centrifugation and lysed in 100 µl lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100). Green fluorescence was analyzed, and transmigration was calculated as percentage of total cells deployed (mean values ± S.D. from quadruplicates).

*Proteolytic digestion of recombinant anx A1*

Recombinant forms of human anx A1 (aa 1-346, aa 47-346, and aa 1-46) were prokaryotically expressed as His6-tagged fusion proteins and purified as described

previously (14). Membrane proteins were prepared from Jurkat cells 18 h after UV irradiation (10 mJ/cm2). Cells were collected by centrifugation, washed in ice-cold PBS supplemented with 10 mM EDTA, and resuspended in 1 ml buffer A containing 20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. The cells were homogenized in a Dounce homogenizer, and the homogenates were cleared at 1,000 x g for 10 min at 4 °C to remove debris. The supernatant was centrifuged at 50,000 x g for 2 h, and the resulting membrane pellet was resuspended in 25 mM Tris-HCl pH 9.0, 2.5 µM ZnCl2. Subsequently, the membrane protein extract of 3 x 107 cells was mixed with 1 µg recombinant human anx A1, incubated for the indicated times at 37°C, and subjected to SDS-PAGE with subsequent immunoblot analysis. Digestion of 1 µg recombinant anx A1 with 100 ng recombinant ADAM10 ectodomain (R&D Systems) was performed accordingly.

*Edman degradation*

40 µg anx A1 (aa 1-346) were mixed with 4 µg ADAM10 ectodomain (R&D Systems) in 25 mM Na-phosphate pH 9.0, 2.5 µM ZnCl2, 0.005% Brij35 and 5 mM $CaCl_2$, and incubated for 4 h at 37°C. Subsequently, the reaction products were applied to N-terminal Edman degradation. 15 µl of sample solution were applied to a TFA-treated glass filter disc coated with 0.75 mg of BioBrene Plus (Applied Biosystems). Filters were not precycled. Sequencing was carried out in a protein sequencer 494A 'Procise' (Applied Biosystems) following the manufacturer's protocols. PTH-amino acids were separated on-line by HPLC using a 2.1 x 250 mm C18 column, detected according to their UV absorbance at 269 nm, and quantitated with reference to a 10 pmol PTH-amino acid standard (all materials from Applied Biosystems). The cleavage site was identified as the newly appearing N-terminal sequence in comparison to the N-terminal sequence of anx A1 that was incubated with heat inactivated (95°C 20 min) ADAM10 ectodomain prior to Edman degradation.

### 6.2.4 Results

*Anx A1 externalization during secondary necrosis is restricted to certain cell types and coincides with anx A1 cleavage*

We have previously shown that anx A1 is externalized during secondary necrosis and provides a dual anti-inflammatory failsafe mechanism by promoting dying cell removal and preventing proinflammatory cytokine production (14). Since this study mainly utilized Jurkat cells, primary PHA lymphoblasts, monocytes, and neutrophils as model systems, we now wanted to analyze, whether anx A1 externalization can also be observed in other

cell types. To this end, different cell lines were stimulated with staurosporine in order to undergo apoptosis followed by secondary necrosis and anx A1 externalization was measured by surface immunostaining and subsequent FACS analysis. Interestingly, exposure of anx A1 was only observed in Jurkat cells, MOLT-4 cells, and primary human PHA lymphoblasts, but not in HuT78, HL60, U937 or THP-1 cells, although staurosporine treatment potently induced apoptosis and secondary necrosis in all cell types employed (Fig. 1A, B). Westernblot analysis was performed to ensure that anx A1 actually was expressed in all the cell types tested. Surprisingly, protein extracts of secondary necrotic cells that had displayed anx A1 exposure (Fig. 1A), revealed an anti-anx A1 reactive band of 36 kDa in addition to the expected full length protein of 38 kDa (Fig. 1C). This band presumably constituted a proteolytic cleavage product of anx A1.

*Anx A1 cleavage occurs on the outer cell surface during secondary but not primary necrosis*

We first analyzed the time course of anx A1 cleavage during staurosporine-mediated apoptosis and secondary necrosis. Interestingly, the appearance of the p36 fragment was observed as late as 9 h after apoptosis induction (Fig. 2A left panel) - a time point, at which the majority of cells had already lost plasma membrane integrity and thus were secondary necrotic (Fig. 2B). In contrast, cleavage of PARP, a prototypical caspase-substrate, was detected already 3 h after apoptosis-induction suggesting that different mechanisms account for the proteolytic processing of these two proteins. Notably, we did not observe cleavage of anx A1 in heat-treated primary necrotic cells (Fig. 2A right panel). From these findings it can be concluded that the proteolytic processing of anx A1 constitutes a late event in the time course of apoptosis occurring after the transition to secondary necrosis. Since we have recently shown that externalization of anx A1 displays similar kinetics, it was conceivable that externalization and cleavage of anx A1 are causally linked events (14). In order to address this issue, we studied the subcellular localization of anx A1 p38 and p36. To this end, secondary necrotic cells were treated with the calcium chelator EGTA to detach externalized, PS-bound anx A1 from the cell surface, and the collected supernatants and cell pellets were applied to SDS-PAGE with subsequent immunoblot analysis. Interestingly, the p36 fragment was only found in the supernatant but not in the residual cell pellet of EGTA-treated cells indicating that it was exclusively located on the outer cell surface (Fig. 2C). Washing with $CaCl_2$ was performed as a control in order to exclude that mechanical cell disruption and subsequent release of intracellular anx A1 accounted for this observation. Here, virtually no anx A1 was found in the supernatant.

The exclusive extracellular localization of anx A1 p36, the kinetics of anx A1 cleavage, and our finding that only protein extracts of anx A1 externalizing cells reveal the p36 fragment allow the conclusion that anx A1 is cleaved after it has been translocated to the outer cell surface during secondary necrosis. Consequently, we wanted to identify the respective protease.

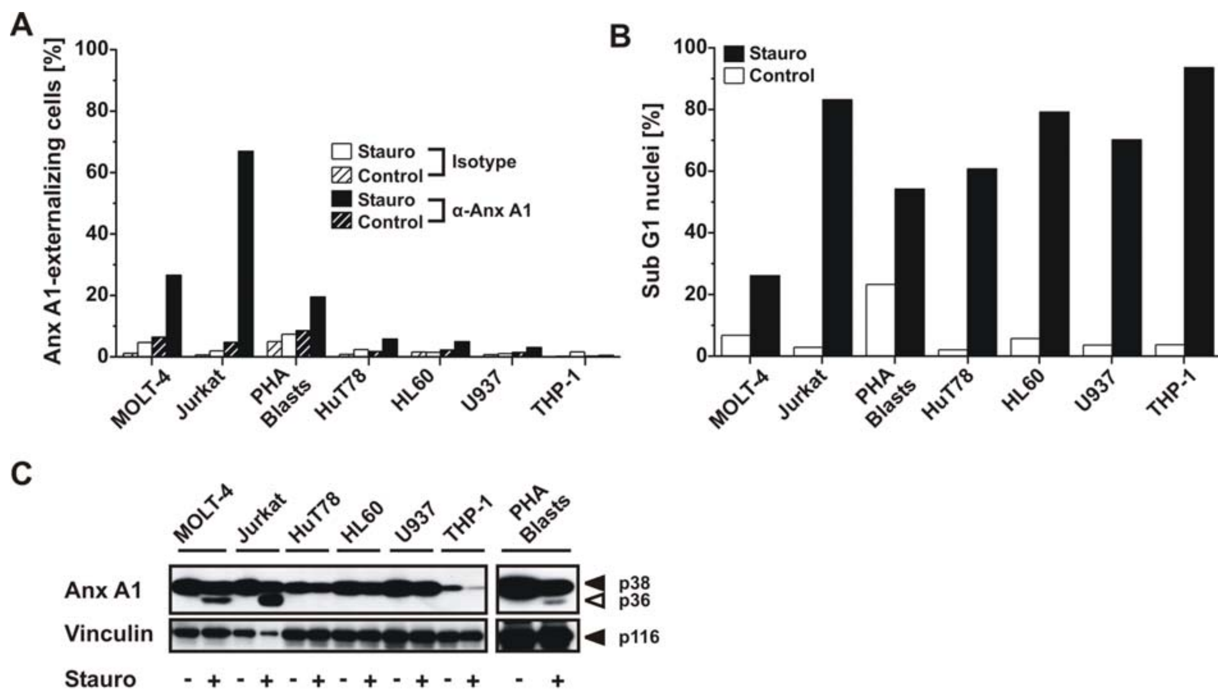



**Figure 1: Anx A1 externalization during secondary necrosis is restricted to certain cell types and coincides with anx A1 cleavage.** (A) *Anx A1 externalization is restricted to certain cell types.* Cells were left untreated or stimulated with 2.5 µM staurosporine (Stauro) for 12 h in order to undergo apoptosis followed by secondary necrosis. Subsequently, cells were stained with anti-anx A1/anti-mouse-IgG-Cy2 and analyzed by flow cytometry. Mouse $IgG_1$ served as isotype control. Mean values of duplicates are shown. (B) *Induction of DNA fragmentation in different cell types.* Cells were stimulated as in (A). After 24 h cell death was measured by flow cytometry and calculated as percentage of subdiploid nuclei (mean values of duplicates). (C) *A 36 kDa cleavage fragment of anx A1 can be observed in anx A1 externalizing cells.* Cells were stimulated as in (A) and protein extracts of 1 x $10^6$ cells per lane were subjected to immunoblot analysis with anti-anx A1 and anti-vinculin antibodies. Vinculin served as a loading control.

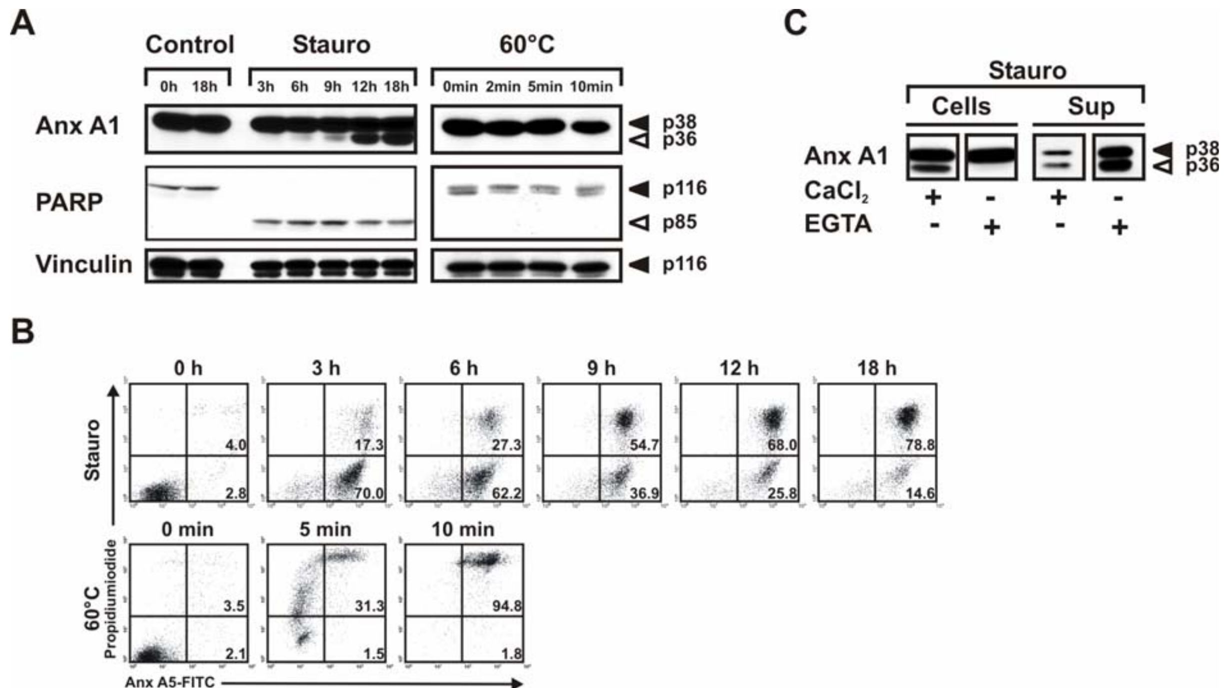



**Figure 2: Anx A1 cleavage occurs on the outer cell surface during secondary but not primary necrosis.** (A) *Anx A1 cleavage occurs during secondary necrosis*. Jurkat cells were heat treated (60°C) or stimulated with 2.5 µM staurosporine for the indicated times in order to undergo primary necrosis or apoptosis followed by secondary necrosis. Subsequently, protein extracts of 1 x $10^6$ cells per lane were separated by SDS-PAGE and subjected to immunoblot analysis using anti-anx A1, -PARP and -vinculin antibodies. (B) *Time course analysis of phosphatidylserine exposure and loss of plasma membrane integrity.* Jurkat cells were stimulated as in (A). Subsequently, exposure of phosphatidylserine and plasma membrane permeability were verified by anx A5-FITC/propidium iodide staining. Representative dotplots of triplicates are shown. (C) *The 36 kDa cleavage fragment of anx A1 is exclusively found on the outside of secondary necrotic cells*. Jurkat cells were stimulated with 2.5 µM staurosporine and incubated for 18 h. Then, cells were washed twice with TBS supplemented with 5 mM $CaCl_2$ or 10 mM EGTA, respectively. Washing supernatants (sup) were pooled and proteins were collected by TCA precipitation. Protein extracts of 1 x $10^6$ washed cells per lane together with TCA precipitated proteins from supernatants were subjected to SDS-PAGE and subsequent immunoblot analysis with anti-anx A1 antibody.

### *Anx A1 cleavage during secondary necrosis occurs downstream of ADAM10*

To date, several reports have described the proteolytic cleavage of anx A1 by different proteases, including human leukocyte elastase and proteinase 3 (6, 7). However, so far no study has addressed the cleavage of anx A1 during secondary necrosis. In order to clarify whether elastase or proteinase 3 are involved in anx A1 processing during secondary necrosis we employed different protease inhibitors: a specific elastase inhibitor and the serine protease inhibitors aprotinin and pefabloc targeting both elastase and proteinase 3. However, neither of these protease inhibitors did significantly interfere with anx A1 cleavage during secondary necrosis as detected by immunoblot analysis (Fig. 3A). Hence, apparently an unrelated protease must be involved.

Intending to narrow down the number of putative protease candidates, we incubated recombinant anx A1 with cell-free supernatants or the membrane fraction of secondary

necrotic cells and subjected the products to SDS-PAGE and immunoblot analysis. Of note, only the co-incubation with the membrane fraction resulted in the generation of anx A1 p36 suggesting that a membrane-resident but not a soluble protease mediated anx A1 cleavage (Fig. 3B). This observation was in accordance with previous reports showing that membrane-bound (metallo-)proteases, including members of the ADAMs family, are activated during apoptosis and perform ectodomain shedding of various transmembrane proteins (22, 23). In order to investigate, whether anx A1 cleavage is indeed mediated by a membrane-localized metalloprotease we next utilized the broadrange metalloprotease inhibitor o-phenanthroline and the more selective matrix metalloprotease (MMP) inhibitor GM6001 targeting various MMPs and ADAMs. Both inhibitors reduced secondary necrosis-associated anx A1 processing in a dose dependent manner (Fig. 3C, D). Remarkably, o-phenanthroline had previously been described to inhibit anx A1 truncation in the context of calcium-induced neutrophil activation (24). Finally, by employing the ADAM10 inhibitor GI 254023X and the ADAM10/17 inhibitor GW 280264X (25) we observed that ADAM10 was crucially involved in anx A1 cleavage, since both inhibitors profoundly mitigated anx A1 processing and GI 254023X almost completely abolished it (Fig. 3E).

To further corroborate the results obtained with the ADAM-inhibitors, we next silenced the expression of ADAM10 and ADAM17 by utilizing different siRNA oligonucleotides. qRT-PCR analysis revealed a knock-down efficiency of approximately 60% for ADAM10 and ADAM17 using different specific siRNAs (Fig. 3F). Most importantly, only secondary necrotic ADAM10- but not ADAM17-silenced cells displayed a convincingly reduced amount of proteolytically processed anx A1 p36 (Fig. 3G). Hence, ADAM10 appeared to be of crucial importance in this scenario.

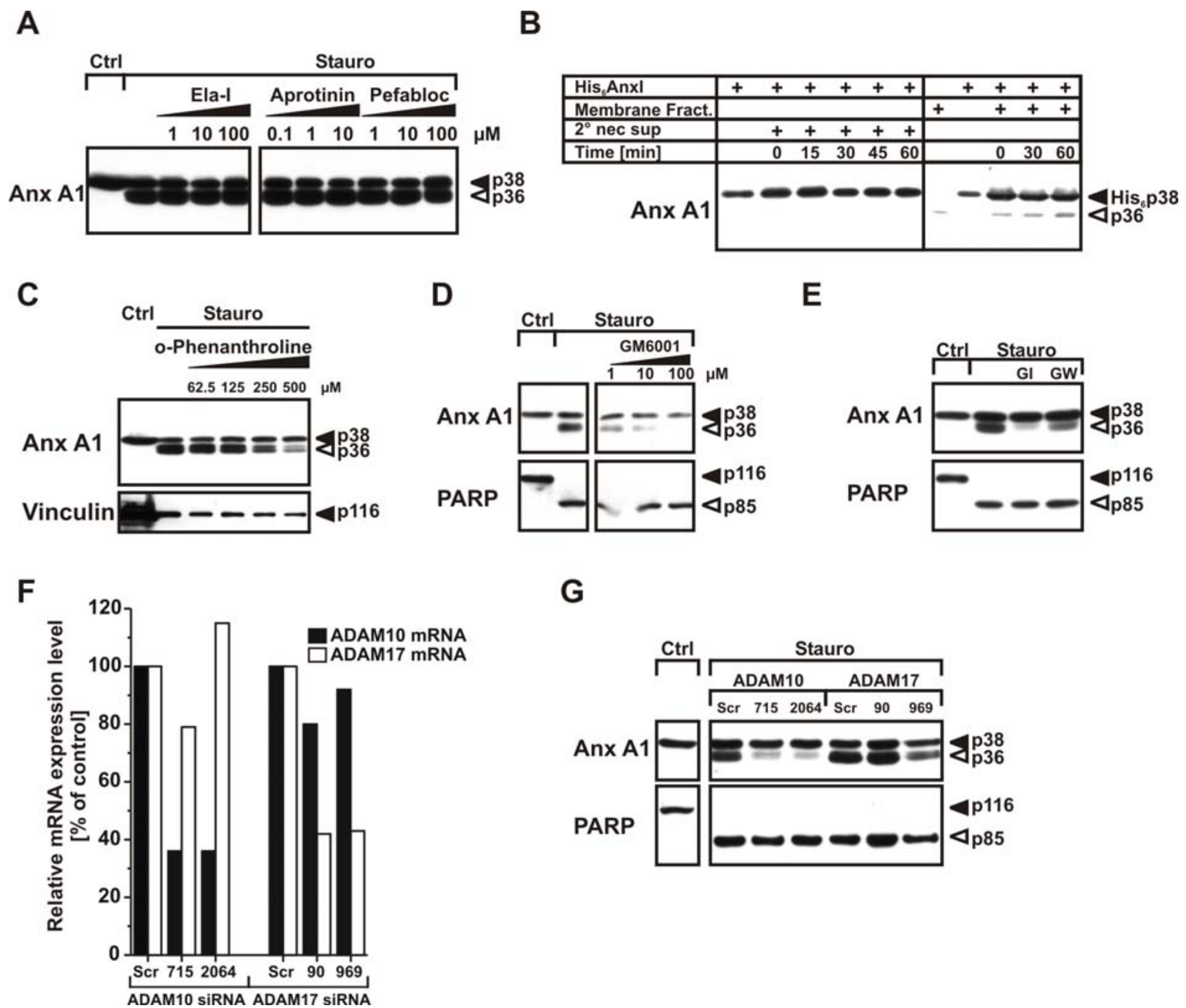



**Figure 3: Anx A1 cleavage is downstream of ADAM10.** (A) *Anx A1 cleavage cannot be blocked by inhibition of elastase or proteinase 3.* Jurkat cells were stimulated with 2.5 µM staurosporine in the absence or presence of elastase inhibitor (Ela-I), aprotinin, or pefabloc for 18 h. Subsequently, cells were lysed and anx A1 processing was monitored by immunoblot analysis. (B) *Anx A1 cleavage is mediated by a membrane-resident protease.* 1 µg of purified recombinant human anx A1 was incubated with the culture supernatant of 4 x $10^6$ secondary necrotic Jurkat cells per ml or the membrane fraction of 3 x $10^7$ secondary necrotic Jurkat cells at 37°C for the indicated times and anx A1 cleavage was examined by immunoblot analysis. (C) *Anx A1 cleavage is blocked by o-phenanthroline.* Jurkat cells were stimulated as in (A) in the absence or presence of the metalloprotease inhibitor o-phenanthroline. Anx A1 processing was detected by immunoblot analysis. Vinculin was used as a loading control. (D) *Addition of the broad-range matrix metalloprotease inhibitor GM 6001 blocks anx A1 cleavage.* Jurkat cells were stimulated as in (A) in the presence of 0-100 µM GM 6001. Afterwards, anx A1 cleavage was monitored by immunoblot analysis. PARP was employed as a loading and apoptosis/secondary necrosis control. (E) *Proteolytic processing of anx A1 can be blocked by the ADAM10 inhibitor GI 254023X.* Jurkat cells were stimulated as in (A) in the absence or presence of 10 µM of the ADAM10 inhibitor GI 254023X (GI) or the ADAM10/17 inhibitor GW 280264X (GW). Subsequently, anti-anx A1 immunoblot analysis was performed with protein extracts as in (D). (F) *Analysis of ADAM10 and ADAM17 knock-down efficiency by qRT-PCR.* Knock-down of ADAM10 and ADAM17 expression was carried out by electroporation of Jurkat cells with 2 different ADAM10 and ADAM17 specific oligonucleotides and a scramble control oligonucleotide as described in 'Materials and Methods'. Total RNA was prepared, reversely transcribed, and the resulting cDNA was used for qRT-PCR as described in 'Materials and Methods'. Relative ADAM10/17 mRNA levels were normalized on the endogenous control ALAS-1, and the ADAM10/17 mRNA level in Jurkat cells that were treated with the scramble control siRNA was set as 100% calibrator. (G) *Anx A1 cleavage is strongly inhibited*

*in ADAM10 silenced cells*. siRNA-mediated knock-down of ADAM10 or ADAM17 expression was performed as in (F). Subsequently, cells were stimulated to undergo secondary necrosis as in (A) and anx A1 cleavage was monitored by immunoblot analysis. PARP served as a loading and apoptosis/secondary necrosis control.

*Anx A1 is directly cleaved by ADAM10 after F7*

The question that needed to be addressed at this point was, whether anx A1 was directly cleaved by ADAM10 or by a protease activated downstream of ADAM10. To this end, purified recombinant anx A1 was incubated with purified ADAM10 and the reaction products were applied to SDS-PAGE and subsequent immunoblot analysis. As shown in Fig. 4B, ADAM10 specifically cleaved anx A1 into p36 and the cleavage was fully abrogated in the presence of the ADAM inhibitor TAPI-2. In order to approximately map the cleavage site, we also digested the purified N-terminal domain as well as an N-terminally truncated mutant of anx A1 with ADAM10 (Fig. 4B, C, D). Proteolytic processing was only observed in case of the anx A1 N-terminus suggesting that the cleavage site must be localized within the first 46 amino acids of anx A1. Subsequent Edman degradation of full length anx A1 cleaved by ADAM10 revealed the cleavage site to be located after F7 (Fig. 4E).

So far, the present study and our previously published observations (14) have shown that certain cell types translocate anx A1 to the outer cell surface during secondary necrosis, where it is proteolytically processed by ADAM10 at F7. This cleavage yields a 36 kDa large core fragment, which stays attached to PS-rich plaques on the cell surface, and an N-terminal peptide of 7 amino acids, which is presumably released. Whereas the cell-bound core fragment has been implicated in the phagocytic removal of the dying cell corpses as well as in the regulation of the post-phagocytic cytoking response (14), the function of the released N-terminal peptide remained elusive.

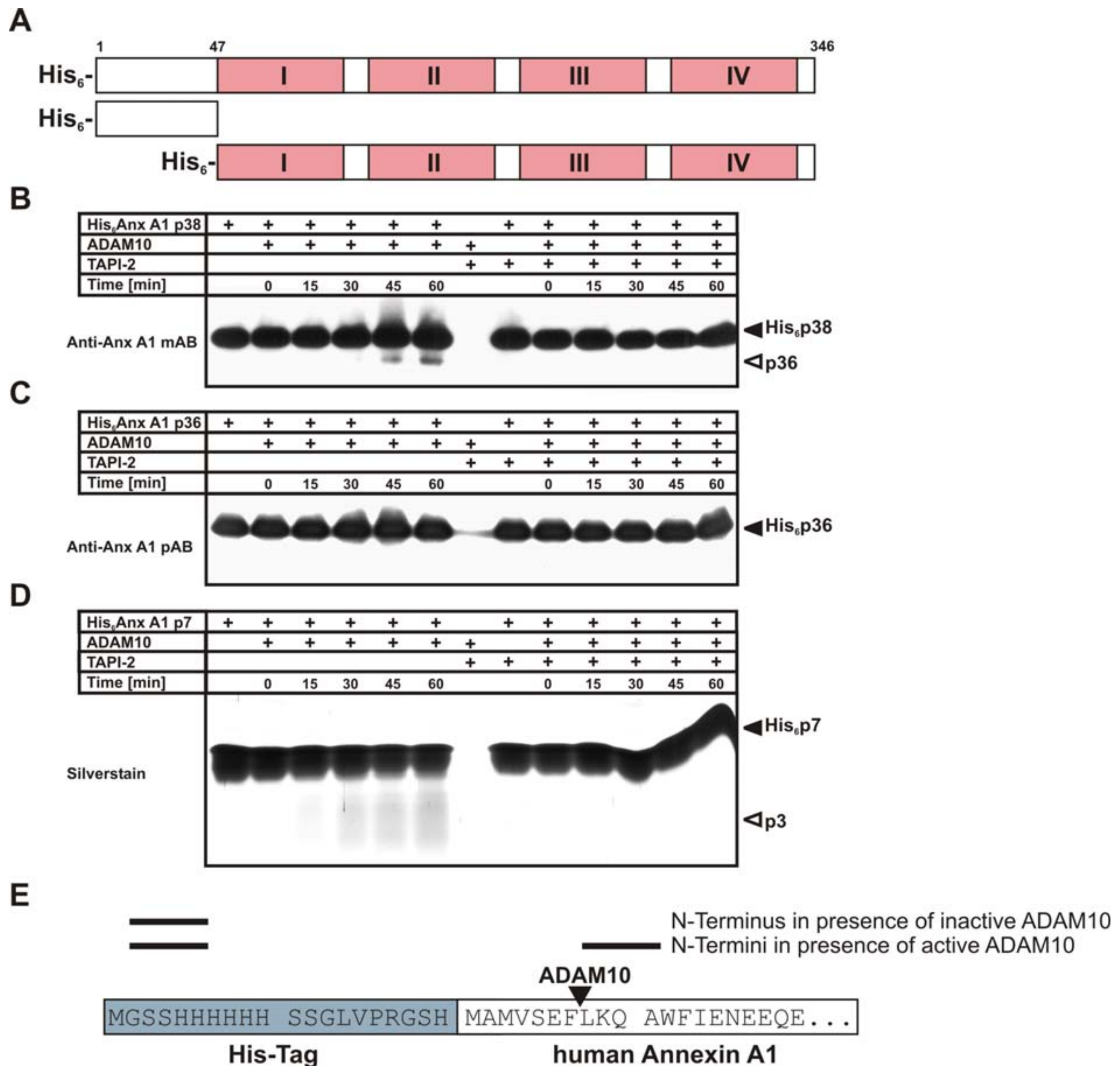



**Figure 4: Anx A1 is directly cleaved by ADAM10 after F$^{7}$.** (A) *Domain structure of different anx A1 constructs*. Arabic numbers depict the amino acid position and annexin repeats are numbered I - IV. (B) *Recombinant human anx A1 (aa 1-346) is processed by recombinant human ADAM10.* 1 µg of recombinant human anx A1 (aa 1-346) was incubated with 100 ng of recombinant human ADAM10 ectodomain in the presence or absence of 100 µM of the matrix metalloprotease inhibitor TAPI-2 at 37°C for the indicated times. Subsequently, anx A1 cleavage was detected by SDS-PAGE and immunoblot analysis with an anti-anx A1 antibody. (C) *Recombinant human anx A1 core domain (aa 47-346) is not cleaved by ADAM10.* Incubation of anx A1 (aa 47-346) with ADAM10 ectodomain was performed as in (B). For immunoblot analysis a polyclonal anti-anx A1 antibody was employed. (D) *The cleavage site of ADAM10 is located within the unique N-terminal domain of anx A1 (aa 1-46).* 1 µg of the recombinant human anx A1 N-terminal domain (aa 1-46) was incubated with ADAM10 as in (B). Cleavage fragments were separated by SDS-PAGE and visualized by subsequent silver staining. (E) *Identification of the ADAM10 cleavage site within the anx A1 N-terminal domain.* Recombinant human anx A1 (aa1-346) was incubated with native or heat-inactivated recombinant human ADAM10 as in (A). Subsequently, the reaction mixture was subjected to N-terminal Edman degradation. The N-terminal sequence newly generated by incubation with active ADAM10 was L$^{8}$KQA.

*The anx A1 N-terminal peptide acts as a 'find-me'-signal of secondary necrotic cells*

For larger peptides derived from the anx A1 N-terminus, including aa 2-26, induction of leukocyte chemotaxis has previously been shown (26). We therefore, sought to analyze, whether this chemotactic activity is preserved within the first seven amino acids, and applied the synthetic peptide to a chemotaxis assay with THP-1 monocytes. Comparable to the classical chemokine MCP-1, anx A1 aa 2-7 stimulated monocyte chemotaxis in a dose-dependent manner (Fig. 5A). The reverse sequence anx A1 (aa 7-2) served as a negative control that displayed profoundly less chemotactic potential. Thus, our results reveal that anx A1 aa 2-7 is endowed with chemotactic activity rendering it likely to be a monocytic attraction signal of secondary necrotic cells.

We tested our hypothesis that the N-terminal anx A1 peptide might be involved in phagocyte recruitment by initially characterizing the biochemical properties of the chemotactic activity in culture supernatants of secondary necrotic cells (Supplemental Fig. 1). Indeed, secondary necrotic cells released monocyte attraction signals, and inhibiting cell death by addition of the poly-caspase inhibitor zVAD-fmk profoundly reduced this release (Supplemental Fig. 1A, B). Checkerboard analysis confirmed that cell migration was due to chemotaxis (directed migration) and not chemokinesis (Supplemental Fig. 1C). In contrast to the classical chemokine SDF-1 the chemotactic activity in supernatants of secondary necrotic cells was heat-stabile and could not be inactivated by incubation at 90°C for 40 min (Supplemental Fig. 1D). Ultrafiltration revealed that substances of an apparent molecular weight of less than 3 kDa accounted for approximately 50% of the chemotactic activity (Supplemental Fig. 1E). Interestingly, Jurkat and MOLT-4 cells, two cell lines externalizing and processing anx A1 during secondary necrosis (Fig. 1), released profoundly more chemotactic activity than non-anx A1-externalizing THP-1 cells. Furthermore, this could be strongly reduced by addition of a commercial protease inhibitor cocktail (Supplemental Fig. 1F). Taken together, the described biochemical characteristics support the notion of peptide-like attraction signals mediating phagocyte recruitment by secondary necrotic cells.

To specifically address the contribution of anx A1-derived peptides in this scenario, we measured monocyte chemotaxis with supernatants of secondary necrotic anx A1 knock-down cells. Intriguingly, we observed a substantial reduction in monocyte attraction (Fig. 5B). Of note, this was not due to the fact that the process of apoptosis or secondary necrosis was impaired by the lack of anx A1 (data not shown and (14)). Instead, anx A1 apparently was crucially required for the generation of secondary necrotic cell-derived 'find-me'-signals supporting our hypothesis that the N-terminal peptide liberated by ADAM10-mediated cleavage of anx A1 might play a role. This was further confirmed by

our observation that supernatants of secondary necrotic cells generated in the presence of GM6001 or GI 254023X displayed a strongly reduced chemotactic potential (Fig. 6A, B). Finally, silencing ADAM10 expression by RNA interference convincingly revealed that a reduction in ADAM10 expression in secondary necrotic cells was paralleled by a robustly decreased extent of phagocyte recruitment (Fig. 6C, D).

In conclusion, our study shows for the first time that anx A1 is externalized and subsequently cleaved by ADAM10 during secondary necrosis, thus liberating an N-terminal peptide of 7 amino acids, which crucially contributes to chemotactic phagocyte recruitment by secondary necrotic cells.

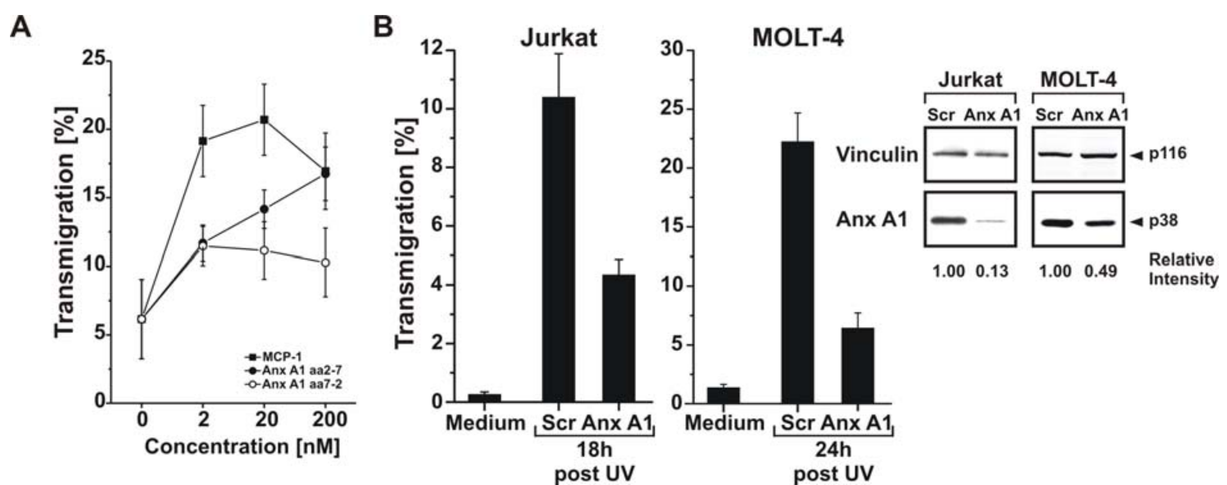



**Figure 5: The anx A1 N-terminus acts as a 'find-me'-signal of secondary necrotic cells.**
(A) *THP-1 monocytes migrate towards anx A1 (aa 2-7).* Anx A1 (aa 2-7) or MCP-1 were added at different concentrations (2-200 nM) to the lower chamber of a double chamber plate. Transmigration assay with THP-1 cells was performed as described under 'Material and Methods'. The reverse sequence of anx A1 (aa 7-2) served as a control. Error bars represent S.D. of quadruplicates. (B) *Release of 'find-me'-signals during secondary necrosis is reduced in anx A1 knock-down cells.* Knock-down of anx A1 expression in Jurkat and MOLT-4 cells was carried out as in Fig. 3G. *Left panel:* On day 5 after the first electroporation apoptosis and subsequent secondary necrosis were induced by UV irradiation (10 mJ/cm$^2$). Cell-free culture supernatants were collected and subjected to a transmigration assay with THP-1 cells as in (A). Mean values + S.D. are shown. *Right panel:* Immunoblot analysis of anx A1-silenced or scramble control cell extracts (10 µg protein per lane) confirmed the efficient down-regulation of anx A1 expression but not the control protein vinculin. The integrated intensity of the anx A1 bands was normalized on the integrated intensity of the vinculin bands and scramble transfected cells were set as 1.00 calibrator.

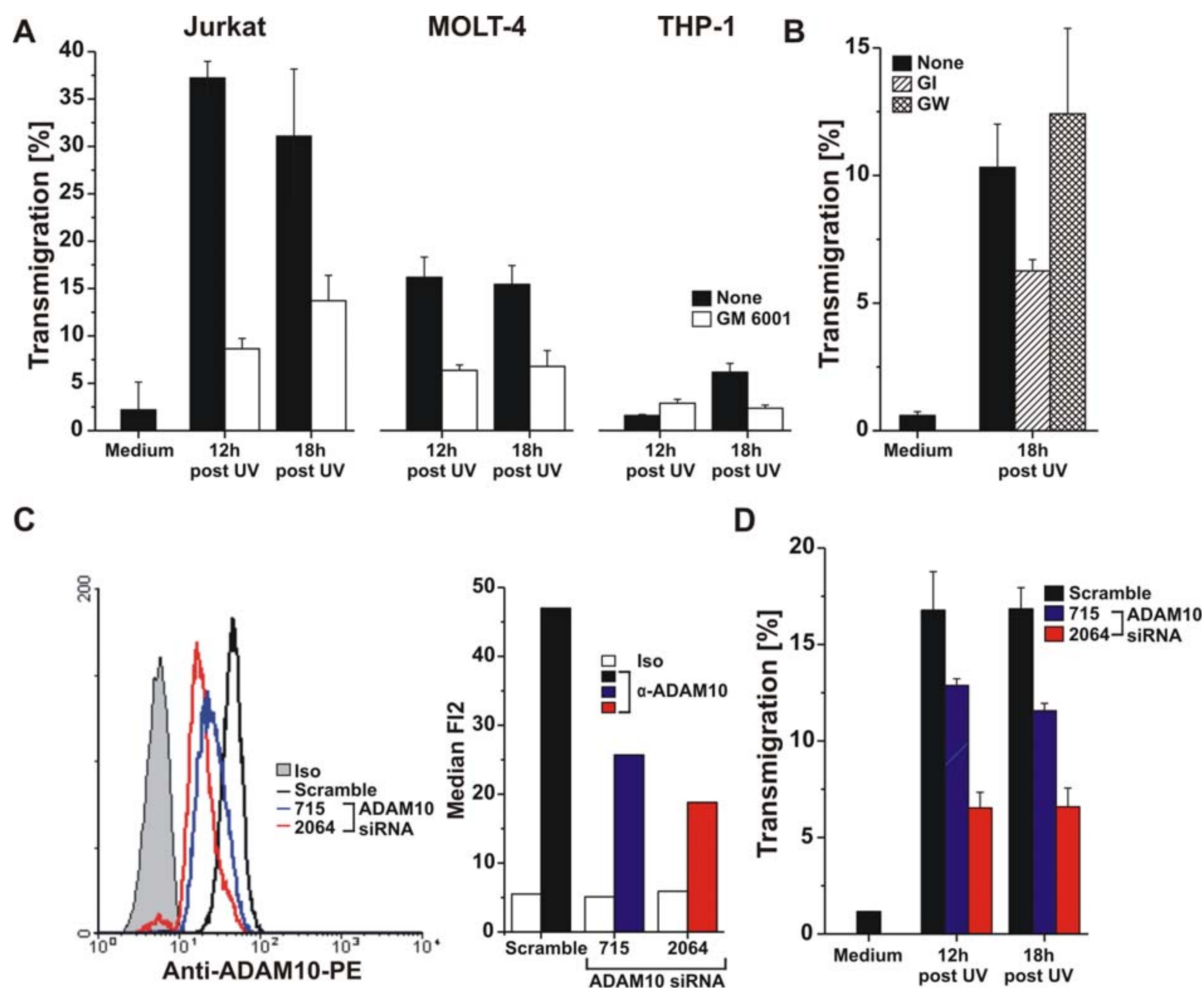



**Figure 6: ADAM10 contributes to the release of 'find-me'-signals from secondary necrotic cells.**

(A) *GM 6001 inhibits the release of monocytic chemoattractants during secondary necrosis.* Jurkat, MOLT-4 and THP-1 cells were UV irradiated and incubated for 12 or 18 h in the presence or absence of the broad-range matrix metalloproteinase inhibitor GM 6001 (100 µM). Cell-free culture supernatants were collected and analyzed for their chemotactic potential as in Fig. 5A. Error bars represent S.D. of quadruplicates. (B) *The release of secondary necrotic cell-derived attraction signals is strongly decreased in the presence of the ADAM10-specific inhibitor GI 254023X.* Jurkat cells were UV irradiated and incubated for 18 h in the absence or presence of 10 µM of the ADAM10 inhibitor GI 254023X (GI) or the ADAM10/17 inhibitor GW 280264X (GW). Supernatants were collected and applied to a transmigration assay with THP-1 cells as in Fig. 5A. Error bars represent S.D. of quadruplicates. (C) *Evaluation of ADAM10 knock-down efficiency by FACS analysis.* Knock-down of ADAM10 expression in Jurkat cells was carried out as in Fig. 3G. On day 5 after the first electroporation cells were fixed, permeabilized and stained with anti-ADAM10-PE antibody to detect ADAM10 expression level by FACS analysis or IgG-2b-PE isotype control. *Left Panel:* Representative histograms are shown. *Right Panel:* Median PE fluorescence of the histograms in the left panel. (D) *The release of monocytic attraction signals is strongly decreased in ADAM10-silenced secondary necrotic cells.* ADAM10-silenced cells were induced to undergo secondary necrosis by UV irradiation. Cell culture supernatants were harvested and analyzed for their chemotactic potential in a transmigration assay with THP-1 cells as in Fig 5A. Mean values + S.D. of quadruplicates are given.

### 6.2.5 Discussion

The development of chronic inflammation and autoimmunity is known to be promoted by defects or delays in the phagocytic clearance of apoptotic cells and the concomitant transition to secondary necrosis (16). Insufficient apoptotic cell removal might be due to direct impairments in the phagocytic process or massive apoptosis overwhelming the phagocytic capacity in the context of acute inflammation, bacterial infections, ischemia, drug-induced hepatitis, or tumor chemo-/radiotherapy (15). During secondary necrosis the plasma membrane becomes permeable and intracellular contents, including cytotoxic metabolic intermediates, hydrolytic enzymes, danger signals, and autoantigens, are released and can stimulate inflammatory and autoimmune reactions.

Our previous studies have demonstrated that anx A1 translocates to the surface of secondary necrotic cells. There, it promotes the phagocytic uptake of dying cells and efficiently dampens the secretion of proinflammatory cytokines by macrophages that have ingested secondary necrotic cells. These findings suggest that anx A1 exposure represents an important anti-inflammatory failsafe mechanism after the transition from apoptosis to secondary necrosis. In our present follow-up study, we have investigated the phenomenon of anx A1 externalization during secondary necrosis in greater detail. Here, we show that after its translocation to the outer leaflet of the plasma membrane anx A1 was proteolytically processed at the cell surface. We could identify ADAM10 as the responsible protease, which cleaved anx A1 within its unique N-terminal domain after F7. Thereby, a small peptide was generated and released from the secondary necrotic cell surface, which in turn induced chemoattraction of monocytes. Hence, anx A1 apparently constitutes a multi-functional protein that acts at different stages and levels of cell death and dying cell clearance.

The results presented here show that externalization of anx A1 during secondary necrosis is not a general, cell type-independent phenomenon but rather restricted to certain cell types. Furthermore, we observed that protein extracts from cells, which exhibited anx A1 externalization in the course of secondary necrosis, contained a proteolytically processed, 36 kDa form of anx A1 in addition to the full length protein of 38 kD. These findings together with our kinetic analyses of anx A1 cleavage and our observation that the cleaved 36 kDa form was exclusively found on the dying cell surface support the conclusion that anx A1 is truncated after its translocation to the cell surface although other reports have shown that anx A1 can also be intracellularly processed by caspase-3 during apoptosis (27). Our pharmacological inhibition and RNA interference studies identified ADAM10 as the responsible protease that cleaved anx A1 during secondary necrosis. ADAM10 is well-known to execute the processing of various transmembrane and

membrane-associated proteins, including MICA, CD46, CD95L, and E-cadherin (22, 28-30). Interestingly, during neutrophil apoptosis ADAM17 has been implicated in the generation of a soluble IL-6 receptor fragment, which governs monocyte/macrophage recruitment for the resolution of inflammation (23). However, in this report the authors did not clearly differentiate whether the cleavage occurs during apoptosis or rather later on, after the transition to secondary necrosis. Nevertheless, with the identification of the anx A1-derived N-terminal peptide our study adds a second 'find-me' signal that is generated in an ADAM10-dependent fashion to the scenario of phagocytic dying cell clearance.

The mechanisms that orchestrate ADAM10 activation during apoptosis and secondary necrosis are far from being understood. Enhanced activity due to an up-regulation of surface expression can be excluded in this context, since we did not detect any significant increase in ADAM10 surface staining during apoptosis or secondary necrosis (Supplemental Fig. 2). However, there is accumulating evidence that high intracellular calcium concentrations and proteolytic maturation are involved (31-33). Since both events might also occur in viable cells or during the early phases of apoptosis (34), the question arises whether anx A1 processing is such a late event in the course of cell death. A feasible explanation would be spatial availability. Anx A1 is an intracellular protein, which is not accessible for the ectoprotease ADAM10. However, after its translocation to the cell surface and the calcium-dependent binding to PS-rich plaques with concomitant exposition of its N-terminal domain (1, 35) the latter becomes available for ADAM10-mediated shedding. Our finding that only anx A1 externalizing cell types displayed anx A1 cleavage during secondary necrosis although all other non-anx A1-externalizing cells expressed ADAM10 on their surface (Supplemental Fig. 3) strongly supports this notion. Therefore, it can be concluded that externalization of anx A1 is the crucial and pace-limiting step in this context.

Sequencing of the cleavage site by Edman degradation has revealed that ADAM10 cleaved anx A1 after F7 within its N-terminal domain. This is the first time that cleavage at this position is reported, although other cleavage sites within the anx A1 N-terminal domain have been described. For instance, during neutrophil activation human leukocyte elastase and proteinase 3 have been shown to process anx A1 at amino acid 26 and between amino acid 29 and 33, respectively (6, 7, 36). The corresponding peptide (aa 2-26) has been implicated in the induction of monocyte chemotaxis, inhibition of neutrophil extravasation and other anti-inflammatory processes, such as reduction of neutrophil-dependent mouse skin edema, inhibition of neutrophil accumulation in zymosan-induced peritonitis, protection from experimentally induced renal ischemia/reperfusion injury, and amelioration of acute carrageenan-induced inflammation (37-39). Intriguingly, it was also

reported that N-terminal anx A1 peptides promote the macrophage-mediated phagocytosis of apoptotic cells (40). The peptide described in the present study (aa 2-7) is much shorter in length. Nevertheless, it convincingly induced monocyte chemotaxis. Most importantly, supernatants of secondary necrotic anx A1 or ADAM10 knock-down cells displayed a profound reduction in their chemotactic potential implying that this peptide essentially contributes to monocyte recruitment by secondary necrotic cells.

The receptors that mediate the effects of anx A1 (aa 2-26) have been identified as members of the formyl-peptide receptor (FPR) family (41-43). Receptor binding and activation has been shown for all three members of the FPR family (26). Notably, THP-1 monocytes that were employed in the present study express FPR1 and FPR2 but not FPR3 at detectable mRNA levels (Suppplemental Fig. 4). However, in the case of FPR1 the crucial motif for receptor binding, activation and desensitization was mapped to anx A1 aa 9-12 (44). This renders FPR1 unlikely to be involved in monocyte migration stimulated by anx A1 aa 2-7, and the responsible receptor remains to be identified in future studies.

Overall, anx A1 represents a protein with various functions at different levels of cell death, dying cell clearance, and anti-inflammation (45). The present study extends this spectrum to a novel function in the context of dying cell removal: Monocyte recruitment by an N-terminal, anx A1-derived peptide that is generated by ADAM10 during secondary necrosis. The multifunctional properties of anx A1 represent attractive targets for novel therapeutic strategies in the context of chronic inflammation and autoimmunity. In this line, local administration of anx A1 or peptides derived from its N-terminus has been reported to alleviate various types of inflammatory reactions (46), and it is tempting to speculate that this might also be a prospective approach for the treatment of inflammatory and autoimmune diseases with known associations to defects in dying cell clearance.

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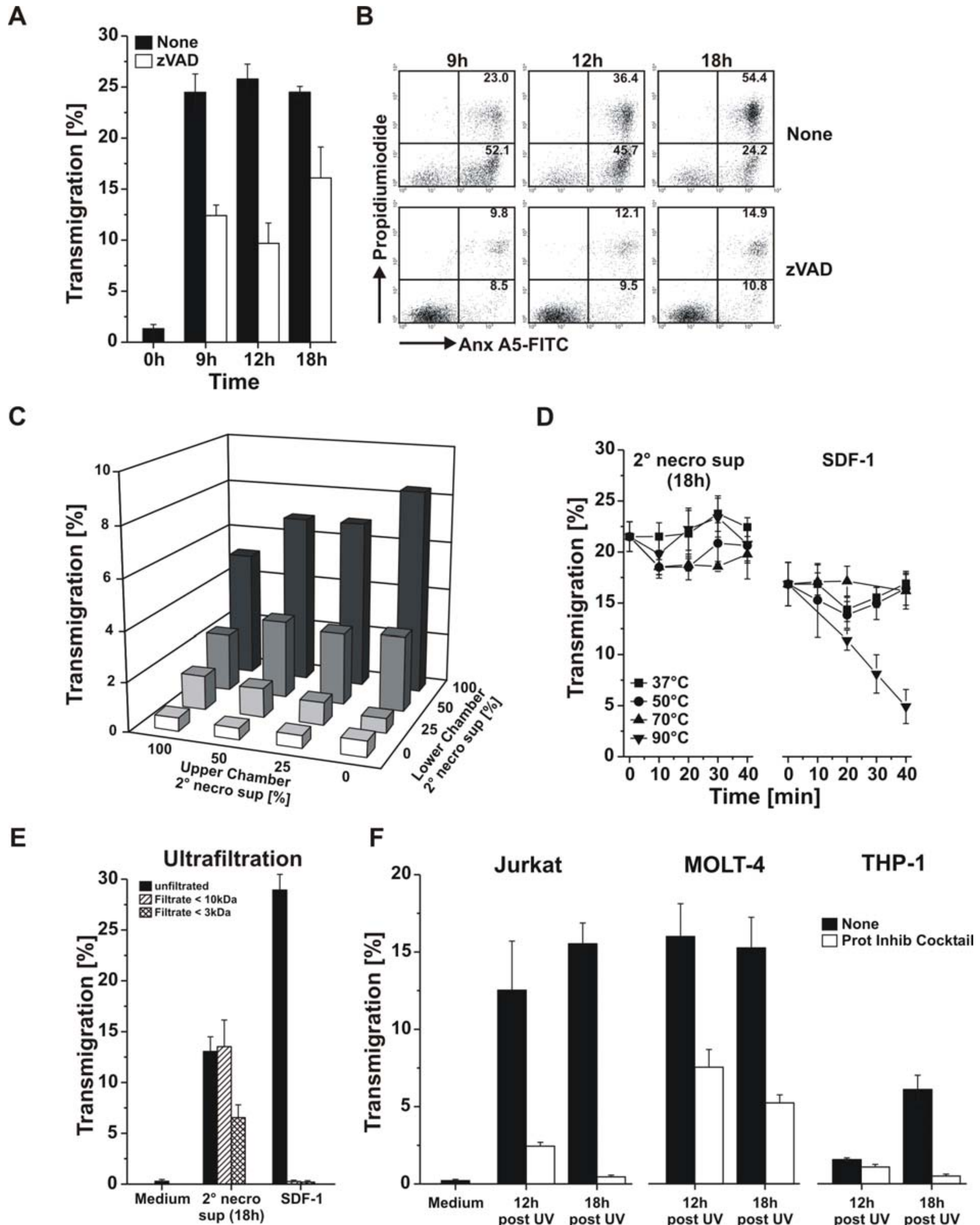
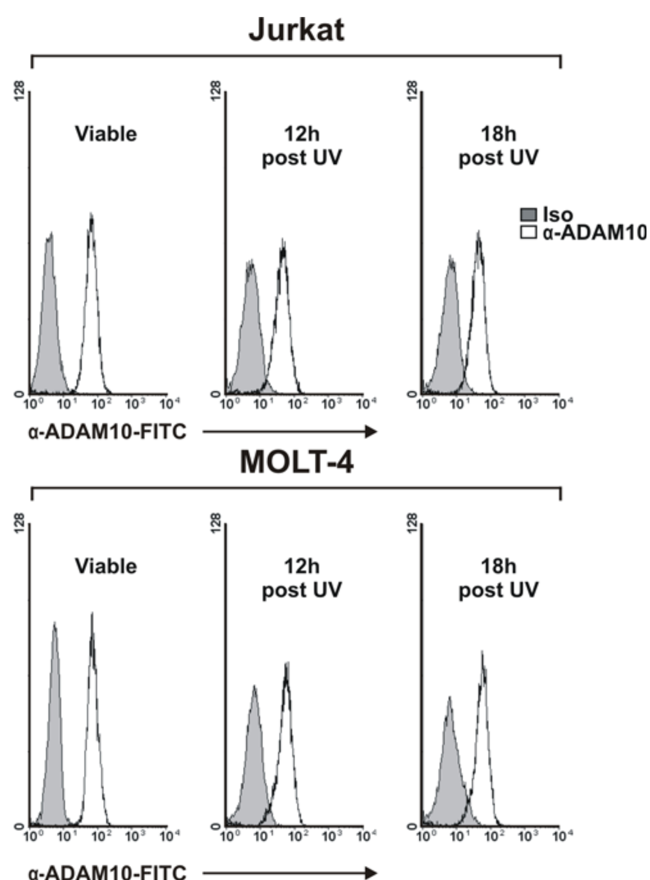
### 6.2.7 Supplemental



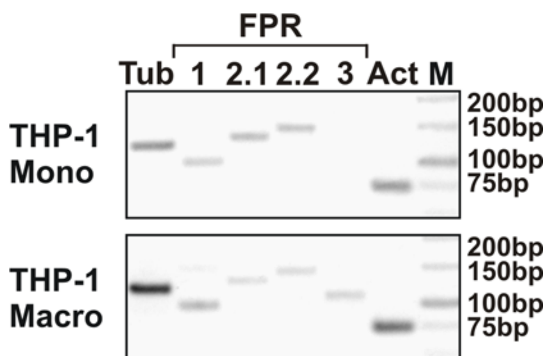

**Supplemental Figure 1: Secondary necrotic cells release small, heat-stable attraction signals, which are produced in a protease-dependent manner.** *(A) Secondary necrotic cells release attraction signals.* Jurkat cells were UV irradiated and incubated for different times (0-18 h) in the presence or absence of zVAD-fmk. Cell culture supernatants were applied to a transmigration assay as described in Fig 5A. Error bars represent S.D. of quadruplicates. *(B) Progression of cell death by detection of PS-externalization and integrity of the plasma membrane.*

Jurkat cells were UV irradiated (10 mJ/cm2) and incubated for the indicated times in order to undergo apoptosis followed by secondary necrosis in the absence or presence of 100 µM zVAD-fmk. Cells were stained with anx A5-FITC to detect PS-externalization and propidium iodide (PI) to distinguish apoptotic cells from secondary necrotic cells by flow cytometry analysis. Representative dot plots of triplicates are shown. *(C) Supernatants of secondary necrotic cells induce monocyte chemotaxis.* Supernatants of secondary necrotic Jurkat cells (18 h after 10 mJ/cm2 UV) were applied at different concentrations to the upper and/or lower chamber of a transmigration plate, and migration of THP-1 cells was assessed as in (A). Means of quadruplicates are shown. *(D) Attraction signals released from secondary necrotic cells are heat stable.* Jurkat cells were UV irradiated to induce secondary necrosis. After 18 h cell culture supernatants were collected and heated at different temperatures for the indicated times. Subsequently, the chemotactic potential was analyzed in a transmigration assay as in (A). SDF-1 served as a heat-labile positive control. Error bars represent S.D. of quadruplicates. *(E) Attraction signals released from secondary necrotic cells are small molecules.* Culture supernatants were generated as described in (A). Thereafter, they were filtrated with different molecular weight cut offs and the chemotactic potential was measured in a transmigration assay. SDF-1 served as a control of approximately 10 kDa. Error bars represent S.D. of quadruplicates. *(F) The release of chemoattractant molecules occurs in a protease-dependent manner.* Supernatants of different secondary necrotic cell lines were produced as depicted in (A) in the presence or absence of a commercial protease inhibitor cocktail, and the chemotactic potential was analyzed in a transmigration assay. Error bars represent S.D. of quadruplicates.




**Supplemental Figure 2: ADAM10 surface expression does not change during secondary necrosis.** Jurkat and MOLT-4 cells were UV irradiated and incubated for 12 or 18 h. Subsequently, non-fixed cells were stained with anti-ADAM10-FITC antibody or the corresponding isotype control to measure ADAM10 surface expression by FACS analysis. Representative histograms of triplicates are shown.

**Supplemental Figure 3: Different cell lines express ADAM10 but not ADAM17 on their cell surface.** Cells were left untreated or were fixed and permeablized as described in 'Materials and Methods'. ADAM10 and ADAM17 expression was detected by FACS analysis using anti-ADAM10- or anti-ADAM17-FITC and the corresponding isotype controls. Representative histograms of triplicates are given.




**Supplemental Figure 4: Detection of FPR transcripts in THP-1 monocytes by RT-PCR.** Total RNA was isolated from THP-1 monocytes and reversely transcribed. 80 ng of the corresponding cDNA were subjected to PCR with exon-boundary spanning primer pairs specific for FPR1, FPR2 transcript variants 1 or 2, FPR3, α-tubulin, and β-actin, and the amplification products were analyzed by agarose gel electrophoresis. cDNA from PMA-differentiated THP-1 macrophages served as a positive control for the amplification of all target cDNAs.

# 7. Abbreviations

| | |
|---|---|
| A | Adenine |
| aa | amino acid |
| α | anti |
| anx A1 | annexin A1 |
| ATP | adenosintriphosphat |
| bp | base pair |
| BSA | bovine serum albumin |
| C | Cytosine |
| C1q | complement protein 1q |
| C3b/bi | complement protein 3b/bi |
| Casp | caspase |
| Caspase | Cystein protease with aspartat cleavage specification |
| CD | *Cluster of Differentiation* |
| cDNA | complementary DNA |
| CED | *Cell Death Protein* |
| *C.elegans* | *Caernohabditis elegans* |
| Da | Dalton |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphat |
| dGTP | deoxyguanosine triphosphat |
| dNTP | deoxynucleoside triphosphat |
| dTTP | deoxythymidine triphosphat |
| DNA | deoxyribonucleic acid |
| DMSO | dimethylsulfoxid |
| DNase | deoxyribonucleic acid-Hydrolase |
| DTT | Dithiothreitol |
| E.coli | *Escherichia coli* |
| EDTA | ethylenediamine tetraacetic acid |
| EGTA | ethylene glycol tetraacetic acid |
| EtOH | ethanol |
| Etopo | Etoposid |
| FACS | fluorescence activated cell sorting |
| Fas | fibroblast associated antigen |
| FCS | fetal calf serum |
| Fl | fluorescence canal |
| FPR | formylpeptide receptor |
| FPRL | FPR-like |
| FU | fluorescence unit |
| G | Guanin |
| g | gram |
| g | acceleration of gravity [m/s$^2$] |
| Gas6 | growth arrest-specific 6 |
| h | hour |
| HEPES | N-2-hydroxyethylpiperazino-N2'-ethanesulfonic acid |
| His | histidin |
| His-Tag | hexahistidin fusion tag |
| HRP | horseradish peroxidase |
| Ig | immune globulin |
| IL | interleukin |
| kb | kilo base |
| kDa | kilo dalton |
| l | liter |
| LB-Medium | Luria Bertani Medium |
| LPC | lysophosphatidylcholin |
| m | mili (10$^{-3}$) |
| m | meter |

| | |
|---|---|
| M | molarity, molar |
| µ | micro (10-6) |
| MFG-E8 | milk-fat-globule-EGF-factor 8 |
| min | minute |
| Mito | Mitomycin C |
| mRNA | messenger RNA |
| n | nano |
| nt | nucleotide |
| ori | replication origin |
| oxLDL | oxidated low-density lipoprotein |
| p50 | protein with a molecular mass of 50 kDa |
| PAA | polyacrylamid |
| PAGE | polyacrylamide gel electrophoresis |
| PARP | poly(ADP-Ribose)-polymerase |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholin |
| PCR | polymerase chain reaction |
| PE | phosphatidylethanolamin |
| PHA | phytohemagglutinin A |
| PI | propidium iodine |
| PMA | phorbolmyristylacetat |
| PMSF | phenylmethylsulfonylfluorid |
| PS | phosphatidylserine |
| PVDF | polyvinylidendifluorid |
| qRT-PCR | quantitative Real Time - PCR |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RNasin | ribonuclease Inhibitor |
| RPMI | Roswell Park Memorial Institut |
| rpm | rotation per minute |
| RT | reverse transcriptation, reverse transcriptase |
| RT | room temperature |
| s | second |
| SDF-1α | Stromal-Cell-derived Factor 1-alpha |
| SDS | sodiumdodecylsulfat |
| SDS-PAGE | SDS-polyacrylamid gel electrophoresis |
| SFM | serum free medium |
| siRNA | small interfering RNA |
| Stauro | Staurosporine |
| T | Thymin |
| TBS | Tris-buffered saline |
| TEMED | N, N, N´, N´, Tetramethylethylendiamin |
| TGF-ß | transforming growth factor ß |
| TNF | tumor necrosis factor |
| TRAIL | TNF-Related Apoptosis Inducing Factor |
| Tris | Tris-(hydroxymethyl)aminoethan |
| Triton X-100 | t-Octylphenoxypolyethoxyethanol, Polyethylenglycol-p-isooctylphenolether |
| TSP-1 | Thrombospondin-1 |
| U | Enzyme unit |
| U | Uracil |
| UV | ultraviolett |
| V | Volt |
| vt | vector |
| zVAD-fmk | Benzyloxycarbonyl-Val-Alex-Asp-fluoromethylketon |

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