Missing in action - the meaning of cell death in tissue damage and inflammation

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Missing in action - the meaning of cell death in tissue damage and inflammation

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Running title

Cell death in homeostasis, inflammation and repair

Summary

Billions of cells die every day in higher organisms as part of the normal process of tissue homeostasis. During special conditions like in development, acute infections, mechanical injuries, and immunity, cell death is a common denominator and it exerts profound effects in the outcome of these scenarios. In order to prevent the accumulation of aged, superfluous, infected, damaged and dead cells, professional phagocytes act in a rapid and efficient manner to clear the battle field and avoid spread of the destruction. Neutrophils are the most abundant effector immune cells that extravasate into tissues and can turn injured tissues into gory battle fields. In peace times neutrophils tend to patrol tissues without provoking inflammatory reactions. We discuss in this review actual and forgotten knowledge about the meaning of cell death during homeostatic processes and drive the attention to the importance of the action of neutrophils during patrolling and for the maintenance or recovery of the homeostatic state once the organism gets attacked or injured, respectively. In this fashion we disclose several disease conditions that arise as collateral damage of physiological responses to death.

Keywords

Clearance, apoptosis, necrosis, neutrophil extracellular traps, pancreatitis, thrombosis

Clearance of apoptotic and necrotic cells

The quiet banquet

The impairment of the clearance of apoptotic cells has been suggested to be responsible for the initiation of systemic autoimmunity in several chronic inflammatory conditions, including the prototypic disease systemic lupus erythematosus (SLE) (1, 2). Apoptosis was initially recognized as a form of programmed cell death occurring during development and considered important to maintain tissue homeostasis (3). Apoptotic cells are engulfed at very early states by phagocytes in an immunologically silent process (4). The silent clearance is warranted since apoptotic cells expose the phospholipid phosphatidylserine (5) while maintaining their plasma membrane integrity, thus preventing release of cellular constituents into the surrounding interstitial tissue (6). Thus the recognition of aged, apoptotic and damaged cells is granted by redundant mechanisms that start with the attraction of phagocytes to the site of demise.

The way to the prey

A plethora of mediators compose a complex and often redundant system including ATP, UTP (7), sphingosine-1-phosphate (8), lysophosphatidylcholine (9) and fractalkine (CX3CL1) (10). The release of such “find me” signals by dying cells stimulates migration and activation of leukocytes (11). The migration of neutrophils toward dying cells is inhibited by secretion of lactoferrin, which acts like a “keep out” signal (12). In case of an enhanced collateral activation of neutrophils, lactoferrin would also contribute to dampening of inflammation by enhancing the aggregation of NETs (neutrophil extracellular traps) (13) (14).

The cutlery for “la grande bouffe”

The distinction between viable and dying cells is mediated by subtle changes in the plasma membrane including the glycocalyx (15) and phospholipids. Phosphatidylserine is a phospholipid present in the inner leaflet of the plasma membrane and gets translocated to the outer leaflet during early stages of apoptosis. Moreover, the increase of the lateral mobility and density of phosphatidylserine on dying cells represents a pivotal event for the recognition as “eat me” signal by phagocytes (16, 17). The receptors on phagocytes directly involved in the recognition of phosphatidylserine are Tim-1, Tim-4, Bai-1 and Stabilin-2 (18). Phosphatidylserine can also be recognized indirectly by phagocytes through several

bridging molecules such as the Milk fat globule EGF factor 8 (MFG-E8) (19), the C-reactive protein (CRP) or growth arrest-specific 6 (Gas-6) (20, 21). The engulfment takes place after the rearrangement of the cytoskeleton to form a phagosome which transfers dead cell cargo to lysosomes in a process referred to as phagosome maturation (7). The molecular pathways involved in the engulfment of apoptotic cells are still a matter of intense research (22).

Siesta - calming down and digest

The consequence of the uptake by phagocytes of apoptotic cells is the secretion of “tolerate me” mediators such as transforming growth factor β (TGF- β) and interleukin-10 (IL-10), which further inhibits the enrollment of inflammatory cells at the site of cell demise. The uptake by alternatively activated resident macrophages of apoptotic cells additionally orchestrates the clearance process by generating 12/15-lipoxygenase-derived products essential for the anti-inflammatory outcome (23). The secretion of pro-inflammatory cytokines like TNF- α , IL-1 β , and IL-12 is also actively downregulated by the phagocytosis of apoptotic cells (4). After engulfment of apoptotic thymocytes, tissue-resident macrophages respond with the inhibition of NF- κ B signaling and repression of inflammatory cytokines, such as IL-12. In the pristane-induced murine lupus this downregulation is mediated by the nuclear receptor Nr4a1 (24). The bottom line is that the clearance of apoptotic cells actively creates an anti-inflammatory milieu wherever cells undergo homeostatic apoptosis (25) (*Fig. 1*).

Fake food

The intrinsic immunosuppressive effect of phosphatidylserine exposure has been hijacked by numerous viruses, microorganisms, and parasites to support establishment and latency of infection. Indeed, several of the most pathogenic viruses, bacteria and parasites reportedly either expose phosphatidylserine or induce phosphatidylserine exposure in bystander host cells (reviewed in (26)). In the infection with *Leishmania major* the development of the clinical disease depends on the presence of phosphatidylserine-exposing dead promastigotes in the virulent inoculum (27) (*Fig. 1*).

In the vicinity of tumors phosphatidylserine is often severely dysregulated and is prone to dampen tumor immunity. Deeper studies of the biology of the apoptotic process allowed the characterization and identification of further recognition domains and ligands like

incompletely processed carbohydrates, galectins, and several lectins (28-30). In these studies it was ascertained that the progression of cell death has broad effects on the outcome of the immune response. The use of AxA5 knockout mice suggested that masking by annexin A5 (AxA5) of phosphatidylserine increases the immune response against tumor cells. Endogenous AxA5 thus acts as a proinflammatory protein to render dying tumor cells immunogenic upon injection (31, 32). The inhibition of phosphatidylserine recognition by exogenously added AxA5 heightened the immunogenicity of irradiated cells (33, 34) (*Fig. 1*).

The orchestration of the immunologic tolerance, rejection and the host responses to dying cells are modulated by specific forms of cell death developing in the living organism (35). Employing an established doxycycline-regulated inducible system that allows triggering various types of cell death directly *in vivo*, the modulatory potential of dead and dying cells was directly evaluated in models of allogeneic tumor/graft rejection (36) and for syngeneic tumors implanted into previously immunized mice (37). In these studies we demonstrated that timing of the release of damage associated molecular pattern (DAMP) crucially determines the outcome of the immune response. All these findings have potential implications in the field of tumor immunology as well as in chronic auto-inflammation (21).

Stodgy food and lousy eaters

When apoptotic cells are not cleared in time, they progress to secondary necrosis losing their membrane integrity and releasing modified autoantigens of nuclear origin together with DAMPs. During apoptosis, a substantial amount of nuclear high mobility group box 1 protein (HMGB1) gets tightly attached to hypoacetylated chromatin, preventing its release (38). In secondary necrosis, HMGB1 is released tightly bound to nucleosomes which in turn contribute to the break of immunological tolerance against DNA (39), the key autoantigen of SLE.

The first hint on a phagocytic defect (for yeast) in patients with SLE was reported back in 1980 (40). *In vitro* differentiated monocyte-derived macrophages from SLE patients showed a decreased and delayed engulfment of autologous apoptotic material (1, 2). They display reduced adherence (41) and a lower expression of the cell adhesion receptor CD44 (42). Adherence and phagocytosis are closely related processes that reflect the clearance capacity of phagocytes. However, an effect of the therapy could not be excluded in these

studies. In order to overcome this, CD34 positive hematopoietic stem cells were isolated from peripheral blood of SLE patients and showed reduced differentiation abilities to professional macrophages (43) suggesting an intrinsic phagocytic defect in a subgroup of patients with SLE.

The autoimmune responses in patients with SLE display attributes of an antigen-driven T cell-dependent immune response, which actually takes place in the germinal centers of secondary lymphoid organs (44, 45). The investigation of the clearance process in the germinal centers of non-autoimmune patients shows tingible body macrophages containing engulfed TUNEL-positive material but seldom free apoptotic nuclei. In some patients with SLE, the amount of tingible body macrophages found in germinal centers was reduced and the TUNEL-positive material was to be found associated with follicular dendritic cells (46). These cells have the ability to expose the dead cell-derived autoantigens to autoreactive B cells that have been accidentally generated by somatic hypermutation. This challenges one checkpoint for the development of tolerance and finally fosters the formation of long-lived auto-reactive plasma cells and consequently the initiation of autoimmunity (47). Anti-dsDNA, anti-histone, anti-Sm, anti-SS-B/La, anti-ribosomal (Ro), and anti-ribonucleoprotein (U1RNP) are produced in patients with SLE and constitute the group of anti-nuclear autoantibodies (ANA) (48). Similarly, anti-phospholipid (49) and anti-C1q autoantibodies are associated with some manifestations of SLE like anti-phospholipid syndrome and lupus nephritis, respectively (50). The existence of ANAs is considered important evidence that a clearance deficiency is directly involved in the initiation of the autoimmune phenomena in SLE (25).

When mice are tablemates

Further evidence can be obtained from several murine models that target clearance-associated proteins and are accompanied by autoantibody production. Knock-out mice for the tyrosine kinase c-Mer (51) and MFG-E8 (19) which recognize apoptotic cells through Gas6 and integrin receptors display a defective phagocytosis of apoptotic cells and develop autoantibodies against DNA and chromatin over time (52, 53). Extracellular chromatin is cleared very efficiently by the action of endonucleases like DNase 1. Loss of function or reduced expression of the *DNase 1* gene has been found in affected kidneys of lupus prone NZBxNZW mice (54). The action of the intracellular DNase II expressed in bone marrow macrophages is extremely important for the prevention of lethal autotoxicity mediated by

type I IFNs (55). When DNase II is deleted and the consequent type I IFN response is avoided by the additional deletion of the IFN type I receptor, the mice develop ANAs and chronic polyarthritis. In this setting the digestion of engulfed nuclei from erythroid precursors is impaired (56) and this might also contribute to the initiation of the autoimmune response by the accumulation of nuclear debris. Natural IgM, along with C1q, facilitates engulfment of dead cells by macrophages in the steady state. Quartier *et al.* showed phagocytic activity of macrophages is reduced 3 to 4 fold in the absence of IgM (57). The role of natural IgM together with further complement proteins during the clearance of apoptotic cells is under debate. It has been postulated that natural IgM antibodies facilitate and promote the silent clearance of apoptotic cells by binding autoantigens with higher avidity than autoreactive IgG antibodies (21). Under the inflammatory conditions after the injection of the alkane oil pristane in mice, IgM seems to contribute to the establishment of the IFN signature (58).

When humans meet at the round table

Genetic defects in the early components of the classical complement pathway are associated with a high risk (>80%) of developing SLE (59). C1q is a strong opsonin for phagocytosis of late apoptotic and necrotic cells and is essential for an effective uptake of degraded chromatin by monocyte-derived phagocytes (60). The rare deficiencies of the complement proteins C2 and C4 are also associated with SLE, glomerulonephritis, and infections (61, 62). Complement receptors and complement inhibitors have also been strongly associated with the development of the IFN signature in SLE (63, 64). Further opsonins like CRP, SAP and the long Pentraxin 3 (PTX3) bind apoptotic material and initiate back-up mechanisms for the clearance of apoptotic cells. In humans, CRP is the main acute phase protein and binds to damaged cell membranes and nuclear components, like U1 snRNP and histones (65, 66). SAP binding to exposed histones contributes to the solubilization of chromatin for further degradation. PTX3 binds to late apoptotic cells and its deficiency leads to a disturbed clearance of apoptotic cells. All these serum proteins are recognized by antibodies from patients with SLE (67-70) meaning that they are present at the crime scene when the breakdown of tolerance has happened.

Spoiled food temptation

The evidence supporting the notion that an impaired clearance of apoptotic cells is involved in the initiation of the autoimmune response has been presented above. The plethora of

factors involved in the etiopathology SLE reflects the complexity of the disease and confirms its multifactorial and multigenetic origin. Considering the chronic nature of the disease one has to assume that the factors conditioning the impaired clearance persist throughout the whole lifespan of the affected individual. This means that remnants of non-cleared apoptotic cells accumulate in tissues of patients with SLE every single day. However, most reports of non-engulfed cellular debris in human tissues besides the lymph nodes (46) date before 1960 and lack modern technology. In the 1950s when the patients were only treated with cortisone, studies reported hematoxylin bodies in cardiac lesions of patients with SLE (71). They were defined as exclusively extracellular, single, clustered, or coalescent masses found specifically in 70-90% of the tissues of mostly untreated patients with SLE (72). They originated from a characteristic alteration of nuclei of mesenchymal cells (73, 74). Regrettably, no association between the presence of this alterations and cell death was made at that time, since the process of apoptosis was first described much later (3). Intensive search for hematoxylin bodies in other diseases were unsuccessful and thus they were considered a reliable criterion for the diagnosis of SLE in necropsies (73, 74). An overview of the studies which systematically looked for hematoxylin bodies is presented in *table 1*. Obtaining similar evidence nowadays is probably impossible since these observations were made in virtually untreated patients and the cause of death was SLE. This warrants that the picture obtained from those necropsies reflected the real situation of the untreated disease, with less than 50% 5-year survival in 1955 (75).

The hangover

Tissue-borne **Secondary NEcrotic Cells** (SNEC) are not only involved in the etiology but also the pathogenesis of SLE. SNEC include proteins that were subjected to caspase cleavage and further post-translational modifications such as phosphorylation, dephosphorylation, ubiquitination, acetylation, and citrullination. SNEC comprise neo-epitopes that challenge the immunological tolerance and foster auto-immunogenicity (76). When anti-nuclear antibodies appear, the formation of immune complexes and the consequent activation of the complement system are programmed.

The inflammation caused by immune complexes is additionally fueled by the involvement of neutrophils, monocytes, and inflammatory macrophages in the clearance process. Sera from patients with SLE often enhance the phagocytosis by macrophages of apoptotic (77) and necrotic cells (78). Anionic phospholipids, particularly phosphatidylserine, are generally

concentrated at the surfaces of apoptotic cells. β 2-glycoprotein-1 and AxA5 bind phosphatidylserine and decorate apoptotic cells. IgG from SLE patients with antiphospholipid syndrome also binds to these proteins on non-cleared late apoptotic cells, enhancing their inflammatory potential by the concurrent secretion of TNF- α (49). Activating complement and engaging Fc receptors, sensitization of SNEC, either with ANA or with anti-opsonin antibodies, shifts their usually silent clearance towards inflammation (79) (*Fig. 1*). Supplementation of healthy blood with IgG from patients with SLE enhances ingestion of SNEC by neutrophils and monocytes (70, 80). The uptake of IgG-sensitized SNEC is tightly coupled with the secretion of various pro-inflammatory cytokines like IL-8, IL-1 β , TNF- β , IL-18 and IFN- α (67, 80).

Several additional players have been identified determining the pathogenicity of autoreactive IgG from patients with SLE and the consequences of its binding to autoantigens. The disease activity of SLE patients positively correlated with the exposure of fucosyl residues on immobilized IgG complexes (81). The non-pathogenic anti- β 2-glycoprotein-1 antibodies of the IgG isotype derived from healthy children display an increased sialylation when compared to pathogenic ones from adults with anti-phospholipid syndrome (82). Pathogenic anti-histone-IgG was mainly present in the non-sialylated fraction and only non-sialylated anti-SNEC IgG promoted SNEC-uptake by neutrophils. The sialylation of specific anti-histone-IgG directly shifted the inflammatory response to SNEC towards a milder form (83). The control of the inflammatory potential of autoantibodies in a model of autoimmune arthritis was governed by the TH17-IL-23 axis. This particular T helper subset regulated the expression of β -galactoside α 2,6-sialyltransferase 1 in antibody-producing B cells and modulated the glycosylation profiles and activity of IgG produced by newly formed plasma cells (84). Immune complexes containing nucleic acids are shuttled by autoantibodies into the intracellular milieu of innate immune cells, triggering inflammatory cytokine responses (80, 85). The overload of phagocytes with nucleic acids has been shown to trigger the exaggerated type I IFN response in DNase2 knockout mice (56). Therefore, we consider nucleic acid containing immune complexes a SLE-specific binary pathogen that precipitates the clinical manifestations of the disease.

Dealing with danger, death and collateral damage - How neutrophil cell death regulates inflammation

Catch and kill: the cellular retiarius at work

The neutrophil granulocyte is known as the prototype gladiator amongst cells. Besides of the classical neutrophil armory phagocytosis (and subsequent destruction of the opponent in phagolysosomes) and degranulation, neutrophils possess an additional mechanism in their arsenal: the formation of neutrophil extracellular traps (NETs). Described initially as a defense mechanism against bacteria and a highly organized and reactive oxygen species (ROS)-dependent active process (86, 87), during the last decade many studies identified numerous triggers (88) and pathways of NET formation (89) and discussed their contents, morphology (90), and the consequences for the NETting cell (91). In this almost Babylonian confusion among published data, the lowest common denominator is that the formation of a NET results in the extrusion of a meshwork of DNA decorated with granular contents into the surrounding extracellular milieu. Thus, NETosis is a particularly dirty kind of cell death with a supposedly inflammatory outcome.

The Janus-faced gladiator: neutrophils in inflammation and autoimmunity

Accordingly, when it comes to autoimmune or autoinflammatory diseases neutrophils were mostly allotted the role of a villain that fuels aberrant immune responses and causes tissue damage. In the development of SLE, the prototypical systemic autoimmune disease, many aspects of neutrophil biology and especially NETs have been implicated (92). NETs are a potential source of SLE autoantigens and are therefore prime candidates for the initiation or enhancement of autoimmunity and tissue damage (Fig. 2). SLE patients produce autoantibodies against many antigens associated with NETs (93, 94). In patients with defective NET degradation levels of anti-NET and anti-dsDNA autoantibodies are further increased and accompanied by renal damage (95). Neutrophils and their NETs have also been connected with development of anti-citrullinated protein antibodies (ACPA) and RA (96), with gout and many other inflammatory disorders (97). Supporting evidence for a pro-inflammatory role of NETs comes from encouraging results of studies using PAD4-inhibitors in lupus and other autoinflammatory conditions (98-101). However, some important findings do not go along with an exclusively pro-inflammatory role of neutrophils and NETs: firstly, human individuals that are unable to form NETs do not fit in this line of arguments. The

outcome of a deficiency of NET-formation in humans can be observed in individuals with chronic granulomatous disease (CGD) and Papillon-Lefèvre syndrome (PLS). In CGD, ROS-dependent NET-formation is impaired due to mutations in the NADPH oxidase complex 2 (102). Individuals with CGD do not only suffer from recurring bacterial and fungal infections, but are also prone to develop autoimmune syndromes (103), which cannot be explained by the inability to clear pathogens. In PLS, NET-formation is compromised by a mutated Cathepsin C that renders all neutrophil serine proteases inactive and therefore impedes chromatin decondensation and canonical NET formation (104, 105). Subjects with PLS are characterized by hyperactivation of neutrophils resulting in exaggerated and non-resolving inflammation, especially in the oral cavity and the skin (106). Interestingly, in experimental lupus and gouty arthritis neutrophil depletion or neutropenia worsens disease ((14, 107)). Also mice strains with normal neutrophil numbers but defects in NET formation have exacerbated and unresolving inflammation. So aside from their given role as the arsonist, neutrophils in general and NETs in particular apparently can have outright anti-inflammatory functions as well.

Yin and Yang: the role of NETs in lupus

In 2012, the group of Mark Shlomchik reported that Nox2-deficient lupus-prone MRL.*Fas*^{lpr} mice developed aggravated lupus, including elevated autoantibody titers and worsened glomerulonephritis (108). These results were recently substantiated and extended by our own studies showing that a mouse strain that carries a mutation in one of the subunits of Nox2 that abrogates Nox2-dependent ROS-production (*Ncf1*^{**} mice) exhibited spontaneous production of hallmark lupus autoantibodies and subclinical inflammation in the kidneys (109). A widely used experimental lupus model develops after intraperitoneal injection of 2,6,10,14-tetramethylpentadecane (pristane), an alkane oil that induces various forms of cell death, among them ROS-dependent formation of NETs (110, 111). Pristane-induced lupus (PIL) was drastically worsened in *Ncf1*^{**} mice (107). Pristane-injected *Ncf1*^{**} mice had higher levels of IgG1 and IgG2a lupus autoantibodies accompanied by increased deposition of IgG and complement C3 in the kidneys that resulted in early proteinuria and premature death. In addition, *Ncf1*^{**} mice also developed strongly exacerbated arthritis after pristane-injection. The absence of ROS impacts cells on many levels and therefore likely has further global influences on lupus than the inability of NET formation (112). We therefore also investigated PIL in mice deficient in peptidyl arginine deiminase 4 (PAD4),

crucial for NET formation in response to calcium-dependent triggers (113). Similar to *Ncf1*^{**} mice, PAD4-deficient mice exhibited higher autoantibody levels and enhanced proteinuria (107). In a parallel study by the Shlomchik lab (114), PAD4-deficiency or pharmacological inhibition of PAD4 did not have any effect on tolerance loss, immune activation, or nephritis in the MRL.*Fas*^{lpr} lupus model.

Interestingly, in our study strongly diminished levels of NETs were found in the pristane-injected peritoneum of both *Ncf1*^{**} and PAD4 knockout mice, showing that pristane-induced NET-formation is augmented by the oxidative burst and citrullination of histones by PAD4. In contrast, we detected a higher percentage of NETs in the blood of *Ncf1*^{**} mice, even before injection of pristane. This was also apparent in patients with SLE and might reflect a pre-primed status of neutrophils derived from *Ncf1*^{**} mice and SLE patients due to subclinical or overt inflammation. Whereas increased presence of NETs in the blood in lupus might fuel the inflammatory process, NET formation in the enclosed pristane-injected peritoneal cavity therefore appears to be an important factor dampening inflammation. The multifaceted role of NETs on autoimmunity and inflammation warrants caution regarding the development of PAD4 inhibitors for the treatment of lupus and inflammatory diseases.

The neutrophil: the acute gout attack's arsonist and firefighter

Also in a mouse model of gouty arthritis reduction of NET formation in *Ncf1*^{**} mice resulted in chronification of the disease (14, 115). Gout is the prototype of an acute self-limiting inflammation in the joints. It results from precipitation and tissue deposition of uric acid in the form of monosodium urate (MSU) crystals, which trigger an inflammatory response accompanied by severe pain, swelling, and erythema. The immense inflammatory potential of acute gout is explained by induction of inflammatory cytokine release by MSU crystals from monocytes and neutrophils. Ingestion of MSU crystals by macrophages results in the intracellular liberation of large amounts of sodium cations that cause osmotic imbalance, drop of intracellular potassium, activation of the NALP3 inflammasome and finally the secretion of the inflammatory cytokines IL-1 β and IL-18 and recruitment of neutrophils (116). Also neutrophils ingest MSU crystals, form NETs (117), and release a variety of inflammatory mediators (e.g., TNF- α and IL-6), neutrophil chemoattractants and activators (e.g., IL-8, MIP-1 α and IP-10) that initially intensify inflammation (118, 119). Remarkably, gouty attacks usually fade within 3–10 days, though MSU crystals are still present in the tissue. Interestingly, antibody-mediated depletion of neutrophils delayed arthritis onset but

also abrogated its spontaneous resolution (14). A similar phenotype was observed in a neutropenic mouse strain (115). Thus, in gouty arthritis neutrophils appear to function as both arsonists and firefighters.

How neutrophils dampen the fire of inflammation

Although neutrophils are usually regarded as pro-inflammatory cells mediating inflammation, evidence is increasing that they can also have anti-inflammatory and immunosuppressive functions. In cancer, immunosuppressive neutrophils are often referred to as granulocytic myeloid-derived suppressor cells (g-MDSC). G-MDSC exist as mature and immature (banded) cells, are able to form NETs (120), and are often of lower densities, co-purifying with PBMCs in density gradient centrifugation, although their granularity is not different from high density neutrophils (121). Of note, low density neutrophils (LDN) with a similar phenotype exert pro-inflammatory functions in the blood of lupus patients due to their high rates of spontaneous NET-formation (122). In cancer however such cells have been shown to suppress T cell responses by releasing arginase-1, a ureohydrolase degrading arginine, which is essential for T cell activation and proliferation (123).

Additional, though indirect evidence that neutrophils often have a regulatory role in inflammation comes from multipotent mesenchymal stromal cells (MSC), a cell type with immunoregulatory and regenerative function. Rather than suppressing neutrophil function, MSCs promote the survival of neutrophils and increase the capacity of the respiratory burst and their activities (124).

Another way of action how neutrophils dampen inflammation is by action of their proteases. Neutrophils contain several protease classes, including cysteine proteases (e.g. cathepsin C, also termed dipeptidyl peptidase I), metalloproteases (e.g. collagenases and gelatinases), and serine proteases. Neutrophil serine proteases (NSP) consist of neutrophil elastase (NE), proteinase 3 (PR3), cathepsin G, and neutrophil serine protease 4 (118). NE, PR3, and cathepsin G are among the most-abundant neutrophil proteins with approximately 1–3 pg per cell. NSPs target a broad array of proteins for modification and degradation. They degrade the extracellular matrix components elastin, laminin, type IV collagen, vitronectin, and fibronectin and enable their migration through the tissue. They also process cytokines and chemokines and their receptors and thus modulate their bioactivity and availability (14). For instance, cleavage by NSPs of TNF- α , IL-2, and IL-6, the cytokine

receptor chain IL-2R- α or the IL-6R 80 kDa chain impair their functionality. In contrast, cleavage of IL-8 by PR3 was shown to yield a more active form of this chemokine (125).

Active pacification: How high density NETs promote resolution of inflammation

The reason for the resolution of inflammation in gout in the persistent presence of high amounts of the initial irritant (MSU crystals) in the tissue was elusive until recently. Interestingly, we observed that MSU crystal-induced ROS-dependent NET-formation is accompanied by massive cytokine- and chemokine-release when only few neutrophils are present. In contrast, when neutrophils are present in high densities, similar to those found after continuous recruitment of granulocytes to an inflammation site, MSU crystal-induced NETs formed dense anti-inflammatory aggregates (aggNETs) (112).

AggNETs are composed of a meshwork of MSU crystals held together by NETs and are reminiscent of the pathognomonical structures of gout: tophi. These tophi are localized macroscopic lesions consisting of aggregates of MSU and several inflammatory cell types, mostly neutrophils and CD68⁺ cells (14, 126). They tend to accumulate on the subchondral bone, the subcutaneous tissue and next to tendons. We showed that tophi isolated from patients with gout share typical characteristics of NETs, such as extra-nuclear chromatin colocalizing with materials from neutrophil granules (112).

Unexpectedly, the concentrations of most cytokines and chemokines in high density cultures of neutrophils were decreased rather than elevated. AggNETs also actively lowered the concentrations of exogenously added inflammatory mediators (14, 115). We then examined the mechanism for this sequestration of cytokines and chemokines. Inhibition of transglutaminases did not show any effect, but blockade of PR3 and NE with elafin, suramin and sivelestat, or incubation with the serine proteinase inhibitor PMSF inhibited degradation by aggNETs of inflammatory mediators.

Aside of DNA and antimicrobial peptides, neutrophil proteases derived from azurophilic (primary) and specific (secondary) granules are a substantial constituent of NETs. Proteome analysis identified NE, cathepsin G, and PR3 in PMA-induced NETs, with NE being the most-abundant non-histone protein in NETs, and cathepsin G and PR3 reaching about 40% and 10% of the amount of NE, respectively (120). NE is also the major contributor to proteolytic NSPs activity within NETs.

We propose that proteolytical degradation of pro-inflammatory mediators by high density NETs interrupts the self-amplifying circle of neutrophil recruitment and activation and is crucial for the resolution of neutrophil-driven inflammation (Fig. 2). In areas of low neutrophil densities (e.g., in blood or in affected tissues during early stages of gout), individual NETting neutrophils trap MSU crystals in an inflammatory manner, releasing their load of pro-inflammatory mediators. In lupus NETs may also serve as an important source of autoantigens, especially if their clearance is insufficient. In areas of high cell densities (e.g., in tissue infiltrates during late stages of gout or the pristane-injected peritoneum), the NETting neutrophils clump and form aggregates. The size and consistency of these aggregates varies depending on the physical and chemical nature of the trigger. Inducers of NET formation may be soluble (e.g., PMA), oily (e.g., pristane) or solid (e.g., crystals). Nevertheless they share the ability to capture and degrade inflammatory mediators by inherent proteases and thus initiate the resolution of inflammation.

The relationship between neutrophil accumulation and NET formation

During the last years the technological progress in two-photon intravital microscopy has enabled observation of neutrophils *in vivo* over extended periods of time. By this means a remarkable neutrophil behavior was discovered that is reminiscent of the swarming of insects and is therefore referred to as neutrophil swarming (127). At sites of tissue injury or infection, groups of neutrophils undergo highly coordinated series of movements, followed by cell accumulation and clustering (128). Neutrophil swarming was observed in several mouse models of tissue wounding, sterile inflammation, and infection and occurs in many mouse organs but also in human PMNs (129). Interestingly, cell death, both in the inflamed surrounding tissue and within the neutrophil cluster itself, seems to strongly influence swarming. The growth and persistence of swarms correlates to the size of the initial tissue damage, likely because the amount of neutrophil-attracting signals released by the damaged tissue is proportional to the size of the injury. Even neutrophil cell death alone can initiate swarming, suggesting that material from the lysed cell may attract other neutrophils (128). It is yet not clear which factors released by dying cells attract further neutrophils and induce swarming and clustering. ATP was suggested as one of these attracting molecules (127, 128). Interestingly, we observed ATP to also augment aggNET formation (14). It is tempting to hypothesize that neutrophil swarming and the formation of high density NETs are interdependent processes, but as for now, the connection between these cellular

behaviors is elusive. A recent study showed that formation of swarms initiated by Alum is followed and augmented by NET formation (130). However, initial swarming did not seem to depend on NET formation. In our study we observed very quick aggregation of neutrophils in the presence of MSU crystals, which was however dependent on the ability to form NETs, since ROS-deficient neutrophils did not aggregate efficiently (14).

The accumulation of neutrophils in swarms and high density NETs is accompanied by advantages and disadvantages: the coordinated cell accumulation sequesters sites of inflammation from viable surrounding tissue and can function as a barrier preventing microbial invaders or other inflammatory triggers from spreading and causing havoc. On the downside, unlimited accumulation in swarms or aggNETs can damage tissue.

NETs clog ducts – occlusion and barrier function by aggNETs

Acute pancreatitis is a devastating disease neglected by most immunologists and cell death scientists (131), even though cell death mechanisms crucially determine the disease outcome and the concomitant fate of the patient (132). Acute pancreatitis may be instigated by diverse mechanisms including toxic (alcohol, drugs) and metabolic dysregulation (hypertriglyceridemia, hypercalcemia). The most common instigator, however, is the obstruction of the common biliopancreatic duct by bile stones or biliary sludge consisting of calcium carbonate, calcium bilirubinate or cholesterol crystals embedded in a viscous extracellular matrix of poorly characterized composition (133). In rare cases, pancreatitis may be due to immune-mediated mechanisms as in so-called “autoimmune pancreatitis” type 1 and 2 (134). The severity of acute pancreatitis varies widely: whereas edematous pancreatitis (80% of all cases) takes a benign course and is treated by mere fluid resuscitation, multi-organ failure and lethality are frequent in necrotizing pancreatitis (135, 136). For clinicians, it is most important to predict severe courses of disease to adapt the modalities of patient care and initiate organ replacement therapies in time. A better pathophysiological understanding of the disease is needed to improve patient care and develop new therapeutic strategies.

Neutrophilic auto-inflammation of the pancreas

In a recent study, we re-evaluated the function of neutrophils in pancreatitis and described a novel mechanism of pancreatitis induction by NETs. We observed pancreatitis and

consequent fibrotic remodeling of the exocrine pancreas in two systems of transgenic IL-17A delivery (137). Delivery of IL-17A alone was capable of stimulating bone marrow granulopoiesis and mobilization of neutrophils to the circulation. Impressively, neutrophils almost selectively infiltrated the pancreas in large quantities without the administration of a specific local trigger. We further noticed that the infiltrating neutrophils did not remain in the interstitial part of the organ but also performed trans-epithelial migration leading to the formation of cellular aggregates inside the ductal lumen of the exocrine system of the pancreas. Interestingly, these intraductal aggregates were immunopositive for citrullinated histone H3, a hallmark of PAD4 activity and a signature of NETs. These observations led us to postulate several hypotheses:

- I) Neutrophils are responsible for IL-17A-induced pancreatitis
- II) The interaction of neutrophils with contents of the pancreatic ductal lumen determines the outcome of disease
- III) Local triggers inside the pancreatic ductal lumen need to be responsible for histone citrullination and aggregated NET formation
- IV) Aggregation of neutrophils inside the ductal lumen strongly impacts epithelial secretory function and flow

In order to test I), we made use of neutrophil depletion strategies in this novel model of IL-17A-induced pancreatitis and, indeed, observed, that neutrophil depletion by anti-Ly6G antibodies abrogated the infiltration of the pancreas by further immune cells and abolished the remodeling of the exocrine pancreas. This highlighted that neutrophils cause the development of pancreatic fibrosis in this model. To approach II), we cultured human PMNs in human pancreatic juice or stimulated these cells with single characteristic components of pancreatic juice. The secretion of the exocrine pancreas is called pancreatic juice and consists of a calcium and bicarbonate-rich fluid further containing precursors of pancreatic digestive enzymes (trypsinogen, chymotrypsinogen), which are usually activated by enteropeptidase in the small intestinal lumen to enable digestive proteolysis. The constitution of the pancreatic juice varies when fasting or after meals and can contain as much as 150mM bicarbonate during the postprandial state to neutralize the pH of the duodenal content after gastric acidification. We observed that neutrophils extruded decondensed chromatin when cultured in human pancreatic juice. The extruded

decondensed chromatin formed macroscopically visible aggregates inside the culture dish. To identify single components of the human pancreatic juice responsible for this effect, we cultured neutrophils in protease-free media with a similar ionic composition as the pancreatic juice. We observed that a disturbed ionic milieu with elevated levels of bicarbonate or reduced amounts of $p\text{CO}_2$ and hence an alkaline-prone pH, respectively, readily induced chromatin decondensation and externalization. We, therefore, identified the “ménage-à-trois” of bicarbonate, $p\text{CO}_2$ and the pH as strong determinants of NET formation (138). Importantly, inhibition of PAD4 reduced the extent of chromatin decondensation by neutrophils cultured in media with slightly elevated bicarbonate levels. As recent studies including our own have implicated crystals as potential instigators of NET formation, we created calcium carbonate precipitations typical of pancreatic concretions. Pancreatic juice is supersaturated with calcium and bicarbonate which facilitates crystalization. Again, when stimulated by calcium carbonate crystals, human neutrophils underwent chromatin decondensation, externalization and aggregate formation. Aggregated NETs were subjected to immunocytochemistry and NE and citrullinated histones were readily detected in the aggregates stimulated by bicarbonate or calcium carbonate crystals. In essence, several components of the pancreatic juice induce neutrophil aggregation and NET formation characterized by citrullinated histone immunopositivity.

Interestingly, IL-17A induced granulocytosis and systemic mobilization of neutrophils in both wildtype and PAD4-deficient mice. However, infiltration of the pancreas by neutrophils and intraductal aggregates of neutrophils were selectively absent in PAD4-deficient mice. Hence, PAD4 activity is crucial for the development of neutrophil-mediated IL-17A-induced pancreatitis.

The pathophysiological chain of events in this model most closely resembles findings from the rare human disease autoimmune pancreatitis type 2, which is characterized by pathognomonic granulocytic epithelial lesions (GELs) (139). This disease is typically associated with chronic inflammatory states like ulcerative colitis and responds readily to corticosteroid therapy (140).

Ductal NETs in biliary pancreatitis

We were, however, intrigued by the concept of trans-epithelial migration of neutrophils and consecutive aggregate formation and suspected a broader relevance of this phenomenon to

the pathogenesis of glandular diseases and specifically pancreatitis. We hypothesized that NETs may be an important constituent of biliary sludge and thereby an inherent cause of acute biliary pancreatitis. NETs in biliary sludge might be an important constituent increasing sludge viscosity and amplifying its obstructive capability (Fig. 2).

Biliary pancreatitis is modeled by some groups by the retrograde infusion of sodium taurocholate into the biliopancreatic duct. Although this can be appreciated as a valid experimental system, one has to keep in mind, that obstructive pancreatitis also occurs without biliary reflux (141). We decided that a modified experimental approach of this technique could determine whether NETs in biliary sludge might contribute to ductal obstruction. As stated above, biliary sludge is characterized by precipitations of either calcium bilirubinate, calcium carbonate or cholesterol crystals. To address this experimentally, we placed calcium carbonate crystals inside the common biliopancreatic duct together with a suspension of murine neutrophils derived from either PAD4 knockout or wild-type mice in order to most closely model biliary sludge. Interestingly, the infusion of both calcium carbonate and neutrophils led to the formation of a duct-occluding mass immunopositive for citrullinated histone H3. The effect of these ductal instillations was most severe when both crystals and neutrophils were instilled, closely mimicking the effect of biliary sludge. Instillation of PAD4-deficient neutrophils reduced the damaging effect on the pancreatic parenchyma as compared to wild-type neutrophils.

Walling off necrotic masses with NETs

Once acute pancreatitis has taken a life-threatening necrotic turn of events, patients suffer from multi-organ failure (e.g. acute respiratory distress syndrome, kidney failure) and the abdominal cavity is characterized by a huge necrotic mass and saponification of the fatty tissue. These disease states may lead to long stays in intensive care units and long-term organ replacement therapies. As pancreatitis-specific causal therapies are lacking, the physicians need to wait for the necrotic mass to be encapsulated and walled-off by wound healing mechanisms of the patient's immune system. This may lead to the formation of walled-off necrotic material or so-called pseudocysts filled with pancreatic or inflammatory secretions (142). We identified NETs in pancreatic collections of patients with necrotizing pancreatitis. We speculated that neutrophils form a primary provisional barrier in response to necrotic tissue by forming NETs. Making use of samples derived from autopsies of two

cases of acute necrotizing pancreatitis we characterized the border of the necrotic tissue by immunohistochemistry (143). A layer of NE- and citrullinated histone H3-positive material surrounded the necrotic core and separated necrotic areas from viable tissue. A further example of the walling off areas of sterile necrotic by NETs was characterized by employing nanodiamonds below the critical size of 50nm. Injection of these particles in tissues causes local sterile necrosis accompanied by a neutrophil-driven initial inflammatory reaction that is promptly controlled by the subsequent formation of aggNETs (13). The aggressive nanodiamonds get sequestered and immobilized by the physical action of the aggNETs and the spreading of the inflammatory reaction gets dampened by the inactivation of chemokines by aggNETs.

This places the neutrophil at the center stage of the pathophysiology of conditions involving tissue necrosis like severe pancreatitis. In essence, neutrophils aim to contain an inflammatory focus. In the case of the walling-off of a necrotic tissue core this may have beneficial functions limiting the spread of toxic cellular debris. However, containment of an inflammatory focus by NETs is also enclosing ductal structures and occlusion by NETs will have detrimental consequences to dependent areas of the organ favoring secretory stasis, premature zymogen activation and consecutive auto-digestion (Fig. 2). In the case of biliary pancreatitis, accidental occlusion of the pancreatic duct may turn the beneficial containment of microcrystals to a toxic waste instigating deadly pancreatitis. In an independent study, Merza et al. have provided important additional clues to the role of NETs in severe acute pancreatitis by treating taurocholate-induced or arginine-induced pancreatitis with systemic DNase I, known to degrade NETs in vitro and in vivo (144). Interestingly, the authors noticed a beneficial effect of DNase-I treatment on severe acute pancreatitis. This effect was both visible locally, as DNase-I treatment reduced local tissue damage but also systemically as reduced lung toxicity was detected. Moreover, in the sera of patients suffering from acute pancreatitis elevated levels of cell-free DNA as well as single NET components were detected, implying that circulating NETs may take part in multi-organ damage in this deadly disease.

The discovery of NETs has substantially altered the concept of extracellular proteolysis, which is enormously relevant also in the field of pancreatic diseases. Usually, serine proteases of both neutrophil or pancreatic origin are subject to inhibition by ubiquitous serine protease inhibitors, such as alpha1-antitrypsin. Binding of serine proteases to DNA

reduces the proteolytical activity of these enzymes. However, DNA-binding also protects serine proteases from alpha1-antitrypsin mediated inhibition and thus permits extracellular proteolysis. This observation may refine future strategies of protease inhibition for the treatment of pancreatitis (145). NET-directed therapies are already a mainstay of therapy of severe pancreatitis: removal of NET-enhanced ductal biliary sludge occlusion by endoscopic retrograde choledochopancreaticography (ERCP) is the backbone of treatment in biliary pancreatitis. Low-molecular weight heparin treatment capable of dismantling circulatory NETs possibly causative for multi-organ failure is provided to almost every single patient in the intensive care unit. Whether additional future NET-targeted therapies may improve the therapeutic outcome will be of outstanding interest.

Taken together, the anatomical localization of NETs strongly determines the respective function of NETs and their role in either containment, restitution, or unintended spread of the disease.

When NETs occlude blood vessels

Canonical hemostasis enables the rapid formation of blood clots at the site of vascular injury, prevents hemorrhage, and involves activation of platelets and fibrin strands. It is initiated by the exposure of subendothelial extracellular matrix (ECM) which binds von Willebrand factor (vWF) and recruits circulating platelets that aggregate and form a plug. In parallel, the extrinsic arm of the coagulation cascade is initiated by tissue factor (TF), a membrane bound endothelial glycoprotein that activates thrombin and fibrin. A plethora of endogenous plasmatic anticoagulants or fibrinolytic proteins tightly control this process. Thrombosis, intravascular blood clotting, is the pathologic counterpart to hemostasis. Thrombosis shares the principle steps of hemostasis, but engages additionally the clotting Factor XII-driven intrinsic coagulation cascade (146). Furthermore, thrombosis closely partners with inflammation and thus may be referred to as “immunothrombosis” (147-149). The contribution of NETs to this cooperation has been documented in several animal models (150). The principal studies analysed the function of NETs in thrombosis by two therapeutic strategies: blocking NETosis by interfering with PAD4 and/or NE activity or infusion of DNase1 or anti-DNA-antibodies to neutralize NETs. In arterial thrombosis triggered by dysfunctional endothelia, a fast and platelet-dependent process, NETs promoted formation of stable occlusive thrombi (98, 151). In venous thrombosis due to

disturbances in blood flow, a slow process driven by fibrin formation, DNase1 infusion or PAD4-deficiency reduced thrombus formation (152-154). In mice prone to develop atherosclerosis, the inhibition of NETosis or the therapeutic DNases reduced atherosclerotic lesions and vascular occlusions (155, 156). Furthermore, a murine model of cancer-induced microvascular thrombosis, infusion of DNase1 reduced systemically blood hypercoagulability (157). The precise mechanism how NETs contribute to vascular occlusions *in vivo* is poorly understood, but *in vitro* experiments suggest that NETs may promote thrombosis by several means. NETs provide a scaffold for the adhesion of blood cells, including platelets and erythrocytes (158). Whether the adhesion to NETs is mediated by entrapment, electrostatic affinity or via a receptor-ligand interaction is elusive. NETs interact with von Willebrand Factor (vWF), an adhesion molecule released by activated endothelia. A protein domain of endothelial vWF interacts with chromatin and may thus immobilize NETs onto the vascular endothelia (159, 160). NETs interact with the plasma clotting factors. NETs stimulate the extrinsic pathway through activating tissue factor (151, 154) inactivating tissue factor pathway inhibitor (151). The intrinsic pathway is initiated by activation of FXII on negatively charged surfaces. NETs bind Factor XII and NET-DNA provides a negatively charged scaffold that activates FXII (154, 161). Furthermore, *in vitro* blood clots with NETs are partially resistant to plasmin-mediated-induced thrombolysis, suggesting that NETs may provide an alternative scaffold to fibrin (158). In summary, NETs contribute to thrombosis possibly through interactions with the vascular endothelium, blood cells, and clotting factors. It is noteworthy that despite the established antithrombotic effect of DNase1 infusion and PAD4 inhibition, neither measure interferes with hemostasis. Thus targeting of NETs may provide safe therapeutic strategy for thrombosis and its associated diseases.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1. Frequency and distribution of hematoxylin bodies in three systematic necropsy studies of patients with Systemic Lupus Erythematosus.

Study	Klemperer 1950		Gueft 1954		Worthington 1959		Total
Patients (n)	35		14		20		69
Disease Controls (n)	n.s.		50		50		100
Organ	n	%	n	%	n	%	Mean %
Heart	21	60	11	79	15	75	77
Kidney	27	77	13	93	12	60	76
LN and Spleen	17	49	12	86	7	35	60
Ovary	13	37	9	64	9	45	55
Serosal Membranes	5	14	6	43	6	30	36
Liver	1	3	3	21	2	10	11
Pancreas	1	3	3	21	1	5	10
Bone Marrow	1	3	2	14		0	9
In two or more organs	24	69	13	93	17	85	82
Overall	32	91	13	93	17	85	90

Figure 1

Figure 1. The progression of the clearance of apoptotic cells determines the final meaning of cell death. Homeostatic apoptosis usually derives in the promotion of self-tolerance in healthy individuals. When find-me and eat-me signals fail, the clearance progress towards the generation of autoimmunity and the precipitation of inflammation. In some cases, the mechanisms of tolerance are hijacked by microorganisms or tumors to evade the action of the immune system.

Figure 2

Figure 2. Consequences of NET formation in sterile diseases

Neutrophil extracellular traps (NETs) form in response to various stimuli (e.g. microparticles (162) or crystals (14), disturbed ionic milieu (138) or necrotic tissue (13, 107)). The aim of neutrophils in sterile diseases is containment of danger and injury. This can be achieved by means of a neutrophil and NET barrier around necrotic tissues or foreign bodies/crystals. NETs come at the price of collateral tissue damage induced by highly basic proteins and NET-bound protease-mediated matrix degradation (163-165), but also contribute to dampening of inflammation by functioning as a cytokine sink (binding and degradation) (14, 115). NETs can aggregate to macroscopically visible clusters (aggNETs) dependent on the amount of neutrophils needed for containment. This may induce the occlusion of ductal structures: blood vessels or glandular ducts, respectively (137, 158). NETs carry antigens of autoantibodies in SLE and small-vessel vasculitis and represent a structural platform for immune complex formation in these rheumatic diseases (93, 95).

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