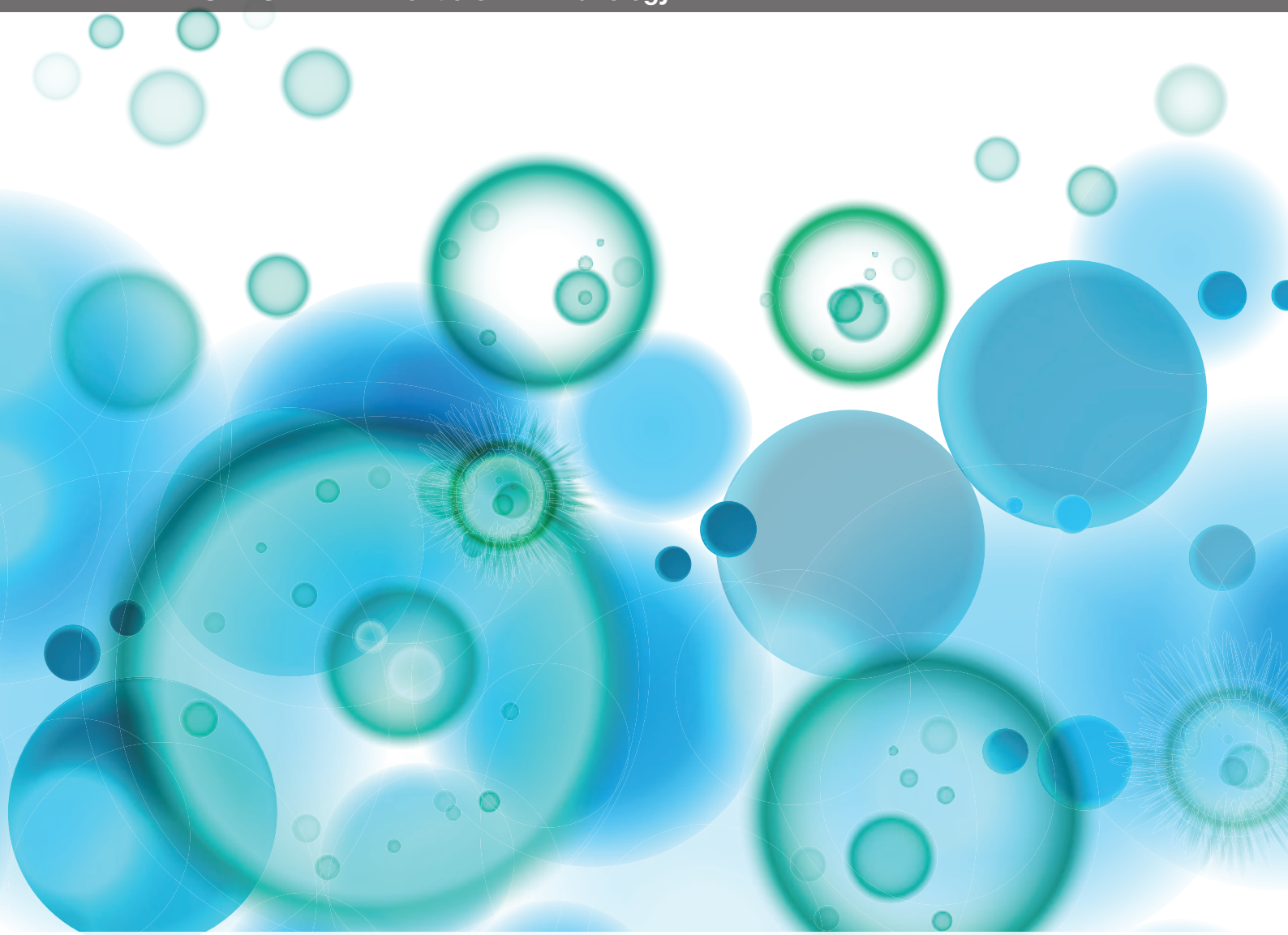
# CHRONIC INFLAMMATION IN CONDITIONS ASSOCIATED WITH A DEFICIENT CLEARANCE OF DYING AND DEAD CELLS, THEIR REMNANTS, AND INTRACELLULAR CONSTITUENTS

EDITED BY: Luis Enrique Muñoz, Christian Berens, Kirsten Lauber, Udo S. Gaipl and Martin Herrmann

PUBLISHED IN: Frontiers in Immunology



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**ISSN 1664-8714**
**ISBN 978-2-88919-601-2**
**DOI 10.3389/978-2-88919-601-2**

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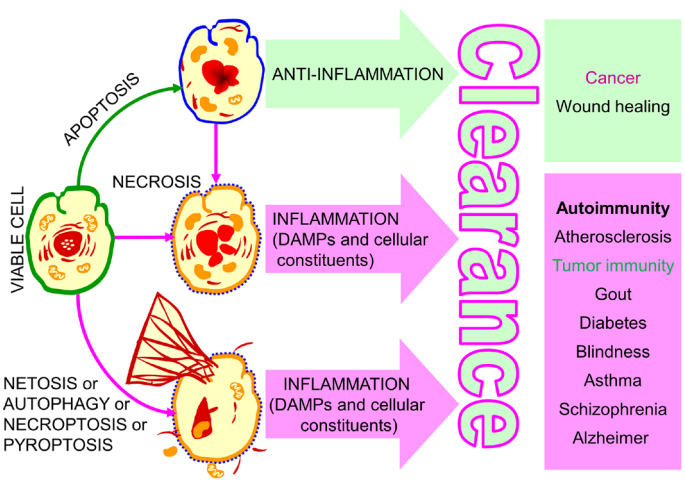
# CHRONIC INFLAMMATION IN CONDITIONS ASSOCIATED WITH A DEFICIENT CLEARANCE OF DYING AND DEAD CELLS, THEIR REMNANTS, AND INTRACELLULAR CONSTITUENTS

Topic Editors:
**Luis Enrique Muñoz,** Friedrich-Alexander University, Germany
**Christian Berens,** Friedrich-Loeffler-Institute, Germany
**Kirsten Lauber,** LMU Munich, Germany
**Udo S. Gaipl,** University Hospital Erlangen, Germany
**Martin Herrmann,** Friedrich-Alexander University, Germany




Efficient clearance of apoptotic cells and cell-remnants is indispensable for normal tissue turnover. Nevertheless, it can be mis-used by transformed cells to foster malignancy. The characterization of various signalling pathways that regulate this complex and evolutionary conserved process has shed light on new pathogenetic mechanisms of many diseases. Impaired clearance not only promotes initiation of autoimmunity and the perpetuation of chronic inflammation, but also may foster anti-tumor immunity. Besides the autoimmune phenotype of chronic inflammatory rheumatoid disorders a plethora of pathologies have been associated with defects in genes involved in clearance.

Figure by Luis Munoz, Christian Berens, Udo Gaipl, Kirsten Lauber and Martin Herrmann.

In multicellular organisms, states with a high degree of tissue turnover like embryogenesis, development, and adult tissue homeostasis need an instantaneous, tightly regulated and immunologically silent clearance of these dying cells to ensure appropriate development of the embryo and adult tissue remodelling. The proper and swift clearance of apoptotic cells is essential to prevent cellular leakage of damage associated molecular patterns (DAMPs) which would lead to the stimulation of inflammatory cytokine responses. In addition to the clearance of apoptotic cells (efferocytosis), backup mechanisms are required to cope with DAMPs (HMGB-1, DNA, RNA, S100 molecules, ATP and adenosine) and other intracellular material (uric acid, intracellular proteins and their aggregates) released from cells, that were not properly cleared and have entered the stage of secondary necrosis. Furthermore, under certain pathologic conditions (e.g. gout, cancer, diabetes) non-apoptotic cell death may transiently occur (NETosis, necroptosis, pyroptosis) which generates material that also has to be cleared to avoid overloading tissues with non-functional cellular waste.

Efficient efferocytosis is therefore indispensable for normal tissue turnover and homeostasis. The characterization of various signalling pathways that regulate this complex and evolutionary conserved process has shed light on new pathogenetic mechanisms of many diseases. Impaired clearance promotes initiation of autoimmunity as well as the perpetuation of chronic inflammation, but may also foster anti-tumor immunity under certain microenvironmental conditions. Immunological tolerance is continuously being challenged by the presence of post-apoptotic remnants in peripheral lymphoid tissues. Besides the autoimmune phenotype of chronic inflammatory rheumatoid disorders a plethora of pathologies have been associated with defects in genes involved in clearance, e.g. atherosclerosis, cancer, gout, diabetes, some forms of blindness, neuropathy, schizophrenia and Alzheimer's disease.

The main goal of this research topic is to collect contributions from various disciplines committed to studying pathogenetic mechanisms of the aforementioned disorders and dealing with alterations in the clearance of dying and dead cells, their remnants, and their constituents that leak out after membrane rupture. Integrating the combined collection of knowledge on efferocytosis and clearance of dead cells and their derived waste from different fields of research in physiology and pathophysiology could improve the molecular understanding of these increasingly prevalent diseases and may ultimately result in new therapeutic strategies.

**Citation:** Luis Enrique Muñoz, Christian Berens, Kirsten Lauber, Udo S. Gaipl and Martin Herrmann, eds. (2015). Chronic inflammation in conditions associated with a deficient clearance of dying and dead cells, their remnants, and intracellular constituents. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-601-2

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frontiers in
IMMUNOLOGY

EDITORIAL
published: 25 March 2015
doi: 10.3389/fimmu.2015.00139

# Apoptotic cell clearance and its role in the origin and resolution of chronic inflammation

***Luis Enrique Muñoz[1]*, Christian Berens[2], Kirsten Lauber[3], Udo S. Gaipl[4] and Martin Herrmann[1]***

[1] *Department of Internal Medicine 3, Rheumatology and Immunology, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany*
[2] *Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Jena, Germany*
[3] *Department of Radiation Oncology, Ludwig-Maximilians-University Munich, Munich, Germany*
[4] *Department of Radiation Oncology, University Hospital Erlangen, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany*
**Correspondence: luis.munoz@uk-erlangen.de*

***Edited and reviewed by:***
*Pietro Ghezzi, Brighton and Sussex Medical School, UK*

**Keywords: clearance, cell death, phagocytosis, chronic, inflammation, cancer, atherosclerosis, autoimmunity**

During normal tissue turnover, innate immune sentinels swiftly clear dying cells in an immunologically silent manner. Large amounts of nuclear chromatin are meticulously kept away from the immune system to prevent inflammation and, eventually, autoimmunity from developing. In case this highly efficient surveillance system is derailed, the unanticipated presence of *post-mortem* remnants in tissues can challenge the otherwise normally ensuing immunological tolerance. Apoptotic cell death is the most natural way to preserve this precious tissue homeostasis. Early recognition and swift clearance of cells undergoing apoptosis ensures the prevention of tissue damage and autoimmune reactions (1). Kimani et al. thoroughly review in this Research Topic, the most recent evidence linking autoimmune diseases and the recognition of apoptotic cells via surface-exposed phosphatidylserine (2).

Besides the autoimmune phenotype of chronic inflammatory rheumatoid disorders, a plethora of pathologies have been associated with defects in genes involved in the clearance of cell remnants from tissues (3, 4). This Research Topic bundles a set of manuscripts describing various ways of how such "uncleared" cell remnants participate in the pathogenesis of chronic inflammatory diseases and also cancer. Improving our knowledge of the immune modulatory language(s) spoken by dying and dead cells and their constituents may prove essential for understanding the key processes involved. Ultimately and hopefully, this may lead to the development of new classes of therapeutic and disease-modifying agents (5).

For example, González and Hidalgo emphasize that it is now possible to take advantage of the huge amount of published evidence on therapeutic modulation of the liver X receptor activity in clearance-associated diseases (6). Notably, pharmacological regulation of such nuclear factors, which are activated upon recognition of dying cells, may enhance the ability of macrophages to clear dead cells and thereby provide additional beneficial effects for treating clearance-related diseases like osteoporosis, rheumatoid arthritis, atherosclerosis, diabetes, and Alzheimer's disease (7–10).

Upon recruitment to sites of acute inflammation, neutrophils respond either with phagocytosis of the inflammatory trigger, degranulation, or with the formation of neutrophil-extracellular-traps (NETs) (11) exposing modified chromatin at the site of the initial injury (12). The nature of this material implies the massive death of neutrophils, and this response is important for both the inactivation of the aggressor and the resolution of the initial inflammation (13). However, how this battlefield is finally cleaned up and cleared has not been studied yet and may surely provide new therapeutic options for autoimmune diseases (12, 14). Intense current research on the immunobiology of this special way of dying called NETosis also promises new therapeutic targets for ameliorating autoinflammation (15, 16). Severe and standard treatment resistant forms of pulmonary inflammation may also profit by novel dual interventions targeting both cell survival and promotion of apoptotic cell clearance by phagocytes as Felton et al. and Szondy et al. summarize in this issue (17, 18). Although one of the multiple mechanisms of action of glucocorticoids is to enhance apoptotic cell clearance by macrophages (19), their long-term use has many side effects that strongly burden chronically diseased patients leading to higher rates of morbidity. Alternatives to classic therapies and specific pathogenesis-targeted therapies are therefore very much welcome.

The many different signals expressed or secreted by apoptotic cells noticeably determine the reaction of the organism to the event triggering death. Any shortcomings in phagocytic clearance, either by impaired clearance, excessive death or any other reason, are invariably related to continuous stimulation of the organism by either pro-inflammatory/destructive or anti-inflammatory/healing signaling. Many chronic inflammatory diseases are driven and somehow modulated by metabolites released from dying cells. For example, Chen et al. present an overview of the various sites of action of nucleotides in inflammatory conditions (20). Intervention at this level may shift the balance toward anti-inflammation, thereby achieving the therapeutic goal more effectively.

In the case of solid tumors, the metabolites and, especially, the danger signals released may serve as biomarkers. Gehrmann et al. nicely demonstrate in this Research Topic that stress response proteins are released already by premalignant conditions of the liver as well as by hepatocellular carcinoma (21). This can be important for prognosis, prediction, and monitoring. The tumor microenvironment, especially directly after anti-cancer treatment, is overflowing with mediators and signals from dead and dying cells. To obtain an efficient anti-tumor immune response, an immune-suppressive microenvironment has to be shifted to an activating one. The

latter might be achieved by rendering the tumor cells immunogenic, namely, by inducing immunogenic tumor cell death forms by standard treatments such as radio- and/or chemotherapy (22). Furthermore, short range danger signals foster leukocyte infiltration into the tumor and initiate an inflammatory response, which is afterwards supplanted by long-range healing and regenerative signals, which then, in contrast, may support tumor proliferation. The review from Willems summarizes evidence documenting the dark side of apoptosis in modulating anti-tumor responses (23). A delicate balance exists between anti-tumor reactions and counteracting immune suppression. In this scenario, the role of tumor associated macrophages as sensors and central orchestrators of tumor-promoting reparatory and anti-inflammatory signals has recently been highlighted by Ford et al. (24). In addition, avoiding tumor repopulation after anti-cancer therapy by considering the immune-suppressive consequences of apoptotic cell clearance should be taken into account as a cautionary premise for each and every anti-cancer treatment (25–28). Of note, inflammatory reactions, DNA damage responses, and cell death forms are highly interconnected (29). Alterations in the clearance of dying and dead cells, their remnants, and their constituents that leak out after membrane rupture are therefore central elements in all inflammatory conditions, starting from its origin and ending in its resolution.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 09 March 2015; accepted: 13 March 2015; published online: 25 March 2015. Citation: Muñoz LE, Berens C, Lauber K, Gaipl US and Herrmann M (2015) Apoptotic cell clearance and its role in the origin and resolution of chronic inflammation. Front. Immunol. **6**:139. doi: 10.3389/fimmu.2015.00139*

*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 10 November 2014
doi: 10.3389/fimmu.2014.00566

# Contribution of defective PS recognition and efferocytosis to chronic inflammation and autoimmunity

**Stanley Gititu Kimani[1], Ke Geng[1], Canan Kasikara[1], Sushil Kumar[1], Ganapathy Sriram[1], Yi Wu[2,3] and Raymond B. Birge[1]***

[1] Department of Biochemistry and Molecular Biology, Rutgers School of Biomedical and Health Sciences – Cancer Center, Newark, NJ, USA
[2] Cyrus Tang Hematology Center, Jiangsu Institute of Hematology, First Affiliated Hospital, Soochow University, Suzhou, China
[3] Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, USA

**Edited by:**
Martin Herrmann, Universitätsklinikum Erlangen, Germany

**Reviewed by:**
Ian Dransfield, University of Edinburgh, UK
Rostyslav Bilyy, Institute of Cell Biology, Ukraine

***Correspondence:**
Raymond B. Birge, Department of Biochemistry and Molecular Biology, Rutgers School of Biomedical and Health Sciences – Cancer Center, 205 South Orange Avenue, Newark, NJ 07103, USA
e-mail: birgera@njms.rutgers.edu

The rapid and efficient clearance of apoptotic cells results in the elimination of auto-antigens and provides a strong anti-inflammatory and immunosuppressive signal to prevent autoimmunity. While professional and non-professional phagocytes utilize a wide array of surface receptors to recognize apoptotic cells, the recognition of phosphatidylserine (PS) on apoptotic cells by PS receptors on phagocytes is the emblematic signal for efferocytosis in metazoans. PS-dependent efferocytosis is associated with the production of anti-inflammatory factors such as IL-10 and TGF-β that function, in part, to maintain tolerance to auto-antigens. In contrast, when apoptotic cells fail to be recognized and processed for degradation, auto-antigens persist, such as self-nucleic acids, which can trigger immune activation leading to autoantibody production and autoimmunity. Despite the fact that genetic mouse models clearly demonstrate that loss of PS receptors can lead to age-dependent auto-immune diseases reminiscent of systemic lupus erythematosus (SLE), the link between PS and defective clearance in chronic inflammation and human autoimmunity is not well delineated. In this perspective, we review emerging questions developing in the field that may be of relevance to SLE and human autoimmunity.

**Keywords: phosphatidylserine, apoptotic cells, scramblases, apoptotic versus non-apoptotic PS externalization, autoimmunity**

## INTRODUCTION

The clearance of apoptotic cells by phagocytic cells (a process now called efferocytosis to distinguish the processing of apoptotic cells from other phagocytic processes) is critically important to maintain homeostasis in multicellular organisms. Efficient efferocytosis not only allows for the removal and degradation of effete and damaged cells, but has an equally important function in the resolution of inflammation by protecting tissue from harmful exposure to the inflammatory and immunogenic contents of dying cells (1–4). There is now considerable genetic evidence supported by mouse knockout studies that failed or delayed efferocytosis results in the release of auto-antigens that can contribute to the etiology of autoimmune diseases such as systemic lupus erythematosus (SLE) (5). In addition, macrophages derived from SLE patients also exhibit defects in efferocytosis (6, 7). Elucidating the genetic basis for defective clearance in relation to human autoimmunity is clearly a topical and important area of research.

Concomitant with caspase activation and cell death, apoptotic cells display a wide array of nascent and modified molecular determinants on their plasma membranes that act as "eat-me" signals for phagocytes. While these determinants result from a combination of re-localized proteins, modified carbohydrates, and from collapse of phospholipid asymmetry at the plasma membrane, the externalization of phosphatidylserine (PS) is arguably the most emblematic event associated with the early phase of apoptotic program (8–10). If apoptotic cells escape immediate clearance, a second wave of late apoptotic cells clearance is mediated by opsonins that includes nuclear materials (11), C1q (12), ficolins (13), and pentraxins (14–16). The late apoptotic cells bound by these opsonins are then recognized and cleared via phagocytic receptors including FcγRIIA, C1q receptor, CR1, CD91, and calreticulin (CRT), helping to avoid inflammation (17, 18). Although our discussion here focuses on cross-interactions between different PS receptors and opsonins, the crosstalk between different recognition systems (such as PS and modified carbohydrates and PS and protein neoepitopes) is likely equally important.

The fact that blockage of PS on the apoptotic cell prevents many of the anti-inflammatory consequences of efferocytosis, combined with observations that knockout of several PS receptors and PS opsonins (soluble factors that link PS on apoptotic cells to receptors) lead to failed efferocytosis, chronic inflammation, and age-dependent autoimmunity (4) has led many investigators to a conceptual framework that externalized PS functions as a dampening platform for negative immune regulation. In this capacity, externalized PS functions both as an "eat-me" signal for efferocytosis, but also as an "inflammo-suppression" signal that promotes tolerance for both immune cells and non-immune bystander cells that come in direct contact with PS externalized membranes (2, 19, 20). Despite convincing evidence as gleaned from knockout studies in mouse, identifying links between defective PS recognition and/or signaling and human autoimmunity has been surprisingly enigmatic (**Table 1**).

**Table 1 | Summary of PS receptors and soluble PS binding proteins and their relationship to autoimmunity in mouse and human systems**.

| Molecule | Function | Mouse | Human |
|---|---|---|---|
| **PS BRIDGING MOLECULES** | | | |
| GAS-6 | Bridging molecule between PS and TAM receptor | Deficiency causes platelet dysfunction and protects against thrombosis (21) | Polymorphism positively associated with cutaneous vasculitis in SLE patients (22) |
| Protein S | Bridging molecule between PS and TAM receptors | Knockout is embryonic lethal (23) | SLE patients have reduced level of circulating protein S (24, 25) |
| MGF-E8 | Bridging molecule between PS and αvβ3/β5 integrins | Deficient mice develops auto-immune disease (26) | Polymorphisms and aberrant splicing reported in some SLE patients (27, 28) |
| C1q | Acts as PS bridging molecule to SCARF1 and CD91/LRP1. C1q also binds annexin A2, A5, and CRT | Deficiency leads to auto-immune diseases (29) | Ninety percent of C1q-deficient individuals develop SLE (30) |
| MBL | Bridging molecule between PS and CD91/LRP1 | Deficiency leads to defective clearance of apoptotic cells but no auto-immune phenotype (31) | Polymorphisms are SLE risk factors (32, 33) |
| High molecular weight kininogen | Bridging molecule between PS and uPAR | NR | NR |
| Thrombospondin | Bridging molecule between PS and CD36 | NR | NR |
| CRT | Binds to PS in a complex with C1q | Knockout is embryonic lethal (34) | NR |
| **PS RECEPTORS** | | | |
| TAM receptors | Indirectly recognize PS via protein S or GAS-6 | Tyro-3$^{KO}$/Axl$^{KO}$/Mer$^{KO}$ triple knockout mice develop auto-immune diseases (35). Mer$^{KO}$ single knockout mice develop progressive SLE-like autoimmunity (36) | Polymorphisms in Mer gene associated with multiple sclerosis susceptibility (37). Increased sMertk in advanced atheromata (38) and SLE (39) |
| Tim-4 | Directly recognize PS | Administration of anti-Tim4 mAb into mice caused auto-antibodies production (40) | NR |
| CD300f | Directly recognize PS | Deficient mice develop a SLE-like disease (41) | NR |
| SCARF1 | Indirectly recognize PS via binding to C1q | Deficient mice developed SLE-like disease (42) | NR |
| Stabilin-1/2 | Directly recognize PS | Deficient mice do not show any SLE-related phenotype (43) | NR |
| BAI-1 | Directly recognize PS | NR | NR |
| RAGE | Directly recognize PS | Deficiency causes impaired phagocytosis but no SLE-related phenotype (44) | Polymorphism associated with SLE and disease severity in lupus nephritis (45) |
| CD91/LRP1 | Indirectly recognize PS via binding to C1q and/or collectins (MBL, SP-A, SP-D) | Deficient mice are embryonic lethal (46) | SLE patients have significantly increased levels of circulating soluble CD91/LRP1 (47) |

*NR, not reported.*

## MICE LACKING PS RECEPTORS ARE PRONE TO LUPUS-LIKE AUTO-IMMUNE CONDITIONS

Over the past decade, a diverse array of PS receptors and soluble PS bridging proteins that link apoptotic cells to phagocytes have been identified (48–50) (**Table 1**). Although this suggests significant redundancy at the biochemical level, PS receptors do not appear to act in a compensatory capacity by loss-of-function. For example, on certain genetic backgrounds, single knockouts of Mer (36), Tim-1 (51), Tim-4 (40), SCARF1 (42), and CD300f (41) all have a common phenotype that include defective apoptotic cell clearance, the subsequent production of auto-antibodies, and SLE-like autoimmunity. Similarly, a knockout of MFG-E8 (26), a PS

opsonin that bridges apoptotic cells to αvβ5 and αvβ3 integrin, also produces a strong SLE-like phenotype. While in some cases dual targeting of PS receptors can compound phenotypic outcomes [for example Tim-4 and MFG-E8 (52) develop autoimmunity at an earlier age, or triple knockout of TAM (Tyro3, Axl, and Mer) (35) have a more potent onset of disease than Mer alone), collectively these data suggest, at least in the mouse, that PS receptors are not functionally redundant. One possible interpretation is that PS receptors, analogously to the immunological synapse for T cell signaling, comprise a multi-protein signaling receptor complex, perhaps akin to a PS phagocytic synapse, where loss-of-function of any single component disrupts the higher order functional unit (53, 54). Several of the known PS receptors, such as αvβ5 integrin and Mertk, are known to synergize in order to activate intracellular signaling pathways such as Rac1 (55, 56) also supporting the idea of receptor crosstalk. However, while attractive to speculate, such a multi-protein structure (aka, the "engulfosome") has not been identified at a biochemical level.

Clearly then, an obvious question is whether the aforementioned PS circuitry fails, or is a genetic risk factor for human auto-immune disease such as SLE. Presently, the answer is still not clear, although of the major PS recognition receptors that give rise to autoimmunity in mice (Mer, Tim-1, Tim-4, SCARF1, and CD300f), their involvement in human autoimmunity is not yet obvious from genetic linkage analysis. Although MFG-E8 mutations have been identified in a small subset of lupus patients (28), and a case-control study of MFG-E8 genetic polymorphisms showed some genetic linkage (27), these events appear to be rare. Likewise in the case of TAMs (Mer) and their ligands, it was shown that in SLE patients, TAM levels do not appear to be compromised (57, 58), and in some patients, serum levels of Mer and TAM ligands actually appear to be elevated (59–61).

The recent studies by Ramirez-Ortiz and colleagues, identifying the scavenger receptor SCARF1 (SREC1, CED-1) as a PS receptor that recognizes a PS in the context of complement component C1q (42) might have relevance to human SLE. *In vivo*, SCARF1 (−/−) mice in develop systemic SLE-like disease, including the generation of auto-antibodies and glomerulonephritis that closely mimics human SLE (42). Interestingly, while SCARF1 was shown to bind via PS, apoptotic cells deficient in C1q were notably impaired in their ability to bind to and activate SCARF1, suggesting the C1q acts as a requisite bridging molecule for PS. In addition to SCARF1, C1q also binds to PS-opsonized CRT (62) on the surface of apoptotic cells (a ligand for CD91/LRP1 on the phagocyte), as well as other PS-binding proteins that include Annexin A5 and Annexin A2 (63). Although genetic deficiency of C1q is quite rare (<100 known cases have been reported), over 90% of these individuals develop SLE (30), and monocytes (64, 65) derived from these patients have impaired ability to clear apoptotic cells suggesting a defect in the apoptotic cell clearance machinery. In addition, apoptotic cells derived from SLE patients also show greatly diminished capacity to bind C1q (66) suggesting one or more of the determinants on the apoptotic cell that bind C1q is also deficient in SLE. Although monocytes isolated from SLE patients showed only a modest decrease in CD91/LRP1 levels, patients with rheumatoid arthritis or SLE showed significantly elevated levels of soluble CD91/LRP1 cleaved by ADAM17 in response to inflammation (47). Possibly related, excessive protease cleavage of Mertk from macrophages has also been linked to inefficient clearance in the development of advanced atheromata (38) and SLE (39). Clearly, it will be of interest to ascertain at the genetic level whether loss-of-function mutations occur at CD91/LRP1 or SCARF1 receptor loci that result in risk associations for human auto-immune diseases.

Taken together, while loss-of-function genetic ablation studies in mouse models clearly show a link between systemic autoimmunity and loss-of-function of PS receptors, translating this biology into human SLE pathology still remains somewhat of a mystery. Future studies should address whether PS receptor biology is arranged differently in humans in comparison to mice PS receptors, allowing for more redundancy, or whether defective PS signaling in human is part of a multi-genic signature that acts as a cohort with other risk factors. Another caveat on relying on expression analysis is that many SLE and auto-immune patients are chronically treated with glucocorticoids and steroids, which may affect the levels of PS receptors or PS-opsonins. For example, Lauber and colleagues showed that MFG-E8 is transcriptionally regulated by dexamethasone, a steroid used to treat the chronic inflammation associated with lupus (67). In addition to MFG-E8, the TAM receptors are also subject to acute regulation by glucocorticoids but in a reciprocal fashion; Mer is up-regulated while Axl is down-regulated following dexamethasone treatment (68). This could also induce a feed-forward mechanism, where dexamethasone-induced increase in Mer levels could increase efferocytosis, which itself further increases Mer by the increased uptake of apoptotic cargo. Internalized apoptotic cells increase ingested cholesterol, which can activate LXR and activate the Mer promoter (69, 70). This idea that corticosteroids mediate their effects by manipulating PS biology might be interrogated via the development of more specific therapeutics for SLE.

Another possible reason for the discrepancy between the studies in mice and the observations in human autoimmunity is that defects in PSR signaling (generated in mouse models) may not be manifested as defects in PSRs or PS-opsonins in human autoimmunity but by mutations in genes involved in the mechanisms upstream such as PS externalization or modification. We explore facets of this hypothesis in the following three sections.

## SCRAMBLASES, FLIPPASES, AND UPSTREAM MECHANISMS OF PS EXPOSURE

While the past decade has shown great strides in elucidating the repertoire of PS receptors that bind to and rely signals from PS on the apoptotic cell to phagocytic receptors, in recent years, there has also been a much greater appreciation for the genes and regulatory circuits that control PS externalization, including the realization that mutations in these genes can lead to pathologies related to dysfunctional PS biology. Novel scramblases and flippases responsible for PS externalization have been enumerated, opening up the possibility that genes that control externalization, and defects therein, may also contribute to chronic inflammation and autoimmunity.

Similar to other lipids, PS is synthesized in the endoplasmic reticulum and golgi apparatus and then transported to the plasma membrane by carrier proteins. Once PS reaches the plasma membrane, it is actively excluded from the extracellular milieu by several complementary enzymes. These enzymes, in part, maintain

membrane asymmetry, with the choline-containing phospholipids; PC and SM predominantly maintained in the outer leaflet, and the amino-phospholipids; PS, PE, and PI predominately on the inner leaflet (71). To maintain PS asymmetry under homeostatic conditions, three main types of enzymes operate at equilibrium, but each can be perturbed during apoptosis and during cell stress. Flippases and Floppases translocate phospholipids from the outer surface to the inner surface and from the inner surface to the outer surface, respectively, and both require ATP for this activity (72). A third, and least understood class of lipid transporters that regulate PS topology are called scramblases, and as their name implies, when activated, collapse membrane asymmetry, and in the context of PS biology, promote the accumulation of PS to the external side of the membrane.

Although phospholipid scramblases do not show selectivity for the phospholipid species or for the direction of movement, the scramblase-mediated exposure of PS has important consequences for several biological events that include coagulation, neurotransmitter release, sperm capacitation, and apoptosis (73). While PS is externalized during both platelet activation and during apoptosis, the recent characterization of two scramblases, Transmembrane protein 16F (TMEM16F) (74, 75) and Xkr8 (76), provide some conceptual relief to this field, highlighting that cells externalize PS through different activation and regulatory mechanisms, but of equal significance, that not all externalized PS has the same biological function.

Transmembrane protein 16F is an eight-transmembrane spanning aminophospholipid scramblase that is critical for the calcium-dependent externalization of PS in activated platelets. In the studies from Nagata and colleagues, these investigators developed a clever FACS sorting approach to characterize a Ba/F3 pro-B cell sub-line that can be trained to respond to sub-threshold concentrations of calcium. After repetitive sorting of PS-positive cells, a Ba/F3 sub-clone that contained a mutated TMEM16F and constitutively scrambled PS was identified (74). Further studies showed that loss of TMEM16F function, either via knockout or through mutation, impairs calcium-dependent PS scramblase activity, and when occurring in platelets, results in their inability to recruit and activate hemostasis factors that include factor V, factor X, and prothrombin to the platelet membrane (75). DNA sequence analysis further showed that Scott syndrome patients, which are characterized by a rare bleeding disorder that have defects on calcium-dependent phospholipid scrambling, carry loss-of-function mutations in both Tmem16 alleles. Functionally, other members of TMEM16 family, including 16C, 16D, 16F, 16G, and 16J are also capable of scrambling PS, but further studies will be required to ascertain whether different family members are specific for different cell types (77).

Notably, the above-mentioned $Ca^{2+}$-stimulated PS externalization induced by TMEM16F is readily reversible upon restoration of $Ca^{2+}$ homeostasis, while the PS externalized during caspase-mediated apoptosis is distinct and separable from TMEM16F, as PS externalization is maintained in apoptotic TMEM16F (−/−) cells treated with Fas-L to induce apoptosis (77). Remarkably, when a mutant TMEM16F was introduced into a mouse lymphoma cell (W3-Ildm) to achieve constitutive PS exposure, these PS-positive tumor cells were not targets of efferocytosis, even by professional DCs, and only became phagocytic competent after treatment with Fas-L to activate caspase 3 (78). These data offer a molecular explanation as to why activated cells, such as during platelet aggregation, T cell activation, and during mast cell degranulation, externalize PS but fail to be engulfed. Conceptually, these data suggest that PS externalization, *per se*, while necessary, is not sufficient to promote clearance (**Figure 1**).

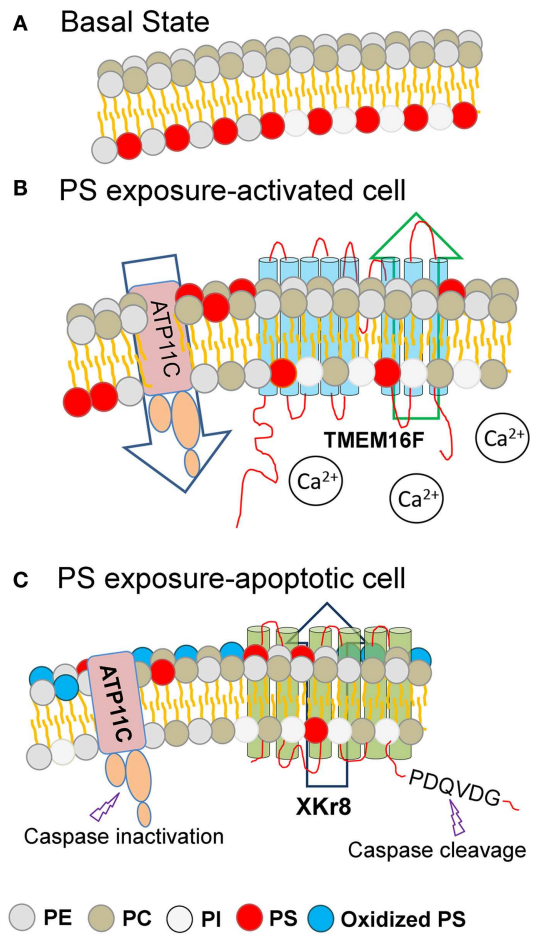


**FIGURE 1 | Different modes of PS externalization by lipid scramblases**. Under basal resting conditions, the amino-phospholipids (PE and PS) are restricted to the inner surface of the plasma membrane **(A)**. During conditions of cell stress or platelet activation **(B)** or during apoptosis **(C)**, membrane asymmetry is lost and PS is externalized to the extrafacial surface (depicted in red). Under basal conditions **(A)**, plasma membrane asymmetry is maintained by the combined activity of cellular flippases and floppases. During cell stress or during platelet activation, intracellular calcium levels rise, resulting in the activation of TMEM16F, and PS exposure to the extracellular leaflet **(B)**. During apoptosis and the activation of caspases, executioner caspases are able to cleave and activate Xkr8, as well as cleave and inactivate ATP11C, resulting in PS exposure to the extracellular leaflet. Since PS externalized PS via TMEM16F and Xkr8/ATP11C are differentially recognized as eat-me signals, it is likely that the density of PS, or the oxidation state of the PS, provide assurance signals for efferocytosis. Key: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; Oxidized PS, oxidized phosphatidylserine.

To identify scramblases associated with apoptosis, Nagata and colleagues used expression cloning to identify scramblases strictly dependent on caspase activity (i.e., inhibited by zFAD-fmk but not dependent on calcium). Based on these screens, a novel scramblase called Xkr8 was identified. Analogous to TMEM16F, over-expression of Xkr8 significantly increased PS exposure, but in stark contrast to TMEM16F, Xkr8 cells that express PS were recognized as an eat-me signal and engulfed. At the molecular level, Xkr8 is cleaved at a DEVD site near its C-termini by caspase 3 and caspase 7 during apoptosis, to activate a PS scramblase activity (76). Xkr8 is a mammalian homolog of the CED8 in *Caenorhabditis elegans* (79) and has an evolutionarily conserved function and is cleaved by CED-3, the homolog of caspase 3, during developmental apoptosis.

Adding complexity to the issue of PS externalization during apoptosis, new studies indicate that a net accumulation of externalized PS is also achieved by a dynamic and systematic interplay between PS scramblases (such as Xkr8) and specific flippases, such as ATP11C (a member of the P4-type ATPase family that redirects PS from the outer membrane back to the inner membrane) (80). Analogous to Xkr8, ATP11C also contains a caspase cleavage site, but when ATP11C is cleaved by active caspases, the Flippase activity is inactivated preventing the return of PS to the inner membrane. Interestingly, when cells express ATP11C with a mutated caspase recognition site, cellular flippase activity remains high, and cells expressing mutant ATP11C do not sustain PS externalization or retain their ability to be engulfed. This presents a highly intricate scenario, whereby caspases can activate Xkr8 and inactivate ATP11C, to increase the steady-state density of externalized PS (**Figure 1**). In contrast, in the non-apoptotic context, high concentration of calcium activates TMEM16, but does not inactivate ATP11C, possibly explaining the reversibility of TMEM16-mediated PS externalization.

Using an LC MS/MS labeling approach to derivatize primary amines on externalized amino-phospholipids (PE and PS), recent studies by Clark et al. found that different molecular species of amino-phospholipids (according to their fatty acyl composition, saturation, length, and oxidative status) were simultaneously externalized during platelet activation versus apoptosis, and revealed an optimal PE fatty acyl chain length that supported coagulation (81). Similar types of MS-based characterization have been reported to define the molecular species of oxidized PS (oxPS) driven by cytochrome c/H202 (82). These kinds of analyses might be revealing to accesses changes in the PS lipidome in SLE patients, or which species of PS are targets of anti-PS or anti-phospholipid antibodies in SLE. Moreover, the recent development of PS reporter lines, such as the generation of chimeric reporter cells to study the PS-dependent dimerization and activation of TAM receptors (Tyro3-γR1, Axl-γR1, and Mer-γR1 cells) (83), or the use of SCARF1 chimeric receptors to access the contribution of PS to C1q signaling (42), would be very useful to explore the functional analysis for PS receptors and to screen apoptotic cells from different cells undergoing apoptosis (normal versus SLE patients). By expanding this kind of analysis, it might be possible to identify if (and how) PS signaling fails during different externalization itineraries. Together, these studies indicate that not all PS externalization is phenotypically equivalent, and relevant to the thesis developed in this perspective, whether the Xkr8/TMEM16F/ATP11c circuit is compromised or genetically linked to SLE or other human autoimmune disorders is an important and timely question in the field.

## OXIDATIVELY MODIFIED PS MAY PROVIDE AN ASSURANCE SIGNAL FOR EFFEROCYTOSIS

The aforementioned discussion between the PS externalization mechanisms of TMEM16F and Xkr8 is instructive, and highlights the fact that PS externalization, *per se*, is not sufficient for efferocytosis. Efferocytosis therefore must require an additional assurance signal, affirming that the cell has passed a caspase-dependent checkpoint and is ready to be engulfed and processed for degradation (84, 85). Although it is likely that other plasma membrane markers act in concert with externalized PS on apoptotic cell, one idea that has gained traction in recent years is that oxPS, generated in a caspase-dependent manner, provides a death-specific marker for PS receptors, marking cells for engulfment (86). oxPS might be expected to change the distribution of PS in the plasma membrane rendering the cell more palatable, or conversely, PS oxidation could serve as a better substrate for PS receptors (i.e., the "altered self" idea) (2).

Although both ideas appear plausible, in support of the latter, it has long been realized that antibodies specific to oxidized phospholipids can block macrophage efferocytosis (87). Moreover, in macrophages, the recognition of apoptotic cells via the scavenger receptor CD36 occurs almost exclusively through interactions with oxPS, and to a lesser extent oxidized PC (oxPC), but not non-oxPS. Interestingly, the specificity of CD36 to oxPS within the apoptotic membranes appears to be mediated by a structurally conserved recognition motif for CD36 that comprises a "sn-2 acyl group with a terminal γ-hydroxy (or oxo)-α, β-unsaturated carbonyl" whereas, the reduction of this acyl chain prevents the oxPS/CD36 receptor activation (88). Other scavenger receptors implicated in apoptotic cell clearance that includes; SRB1, SRA, LOX-1, CD68, and CD14 (2, 89) also appear to selectively recognize the oxidized sn-2 acyl group, suggesting this may be a conserved and universal epitope in the apoptotic program.

In addition to scavenger receptors, recent studies also show that some of the conventional PS-binding proteins and receptors, such as GAS-6 and BAI-1, preferentially interact with oxPS, although in the same study, it was also shown that non-oxPS preferentially bound CXCL16 and Tim-4 (90), suggesting variations on this theme. Although previous studies showed that the peroxidase function of caspase 3 could directly oxidize PS, PS can be oxidized during inflammation as a result of enhanced lipid peroxidation (88). The fact that various oxPS species may alter the repertoire and/or change the affinities of PS toward scavenger receptors and PS receptors provides an impetus to better understand the molecular basis of PS oxidation.

It is also noteworthy that oxysterols and oxPS can also indirectly impinge on efferocytosis. For example, the engulfment of apoptotic cells brings in large amounts of cellular lipids, including the oxidized lipids alluded to above, into the intracellular compartments of the phagocyte. Elegant studies have shown that these internalized lipids can activate PPAR-δ receptors (91) and the nuclear receptor LXR in macrophages (69), to induce engulfment

receptors such as Mer and C1q. In mice, genetic ablation of PPAR-δ results in impaired apoptotic cell clearance and SLE-like disease (92), although the significance to human lupus still remains to be determined.

## LYSO-PS, A UNIQUE FORM OF PS, BINDS DISTINCT RECEPTORS AND IS INVOLVED IN THE CLEARANCE OF NON-APOPTOTIC NEUTROPHILS

Finally, in addition to (i) the modes of externalization, (ii) whether PS is covalently oxidized, and (iii) whether a PS receptor is available to bind exposed PS on the surface of the apoptotic cell, under certain circumstances PS can also be hydrolyzed under oxidative conditions by a PS-specific phospholipase (PS-$PLA_1$) (93–95) to generate lyso-PS, a deacylated form of PS that serves as an endogenous anti-inflammatory mediator. Although lyso-PS can stimulate efferocytosis under certain conditions (96), this form of PS remarkably also stimulates the uptake of live cells, and has been implicated in the clearance of activated and aged live neutrophils in anticipation for the resolution of inflammation. Despite that PS and lyso-PS have the same anionic head group, lyso-PS does not bind conventional PS receptors such as TAMs and TIMs, but instead interacts with two G-protein coupled receptors, GPR34 and G2A (97), which are linked to novel anti-inflammatory molecules such as PGE2.

## LESSONS FROM BLOCKING PS IN CANCER MODELS

In recent years, the idea that PS serves as a tolerogenic and global immunosuppressive checkpoint has been therapeutically exploited by the generation of anti-PS antibodies for cancer immunotherapy. These studies show that systemic treatment of Bavituximab (which recognizes a complex of β2-glycoprotein and PS), can activate immune checkpoints, and drive the polarization of macrophages from M2 to M1 and the activation of immature DCs to antigen presenting cells, while decreasing MDSCs and Tregs in tumor-bearing mice (98). As such, this pre-clinical finding has an unanticipated consequence to ask whether blocking PS is sufficient to induce autoimmunity. While the answers are not completely clear, the available pre-clinical and clinical biosafety studies using acute rather than chronic dosing regiments of Bavituximab (anti-PS antibodies), suggest that anti-PS antibodies are well tolerated and do not produce systemic autoimmunity or pulmonary thrombosis (99). Furthermore, vaccinating mice with apoptotic RMA lymphoma cells pre-treated with Annexin-V attenuated the ability of mice to reject a challenge with live RMA lymphoma cells (100). Whether systemic anti-PS treatment exacerbates auto-immune responses in lupus-prone individuals, or in individuals with anti-phospholipid antibody (syndrome), has not been investigated. It will be of interest to identify if patients that develop anti-PS antibodies in SLE might have naturally occurring decreased metastatic burden. Together, these data suggest that blockage of PS, *per se*, may not be causal for the development of lupus, but nonetheless re-activates specific arms of the immune response, which may be fortuitously exploited where immunosuppressive mechanisms operate within the tumor microenvironment. Future studies, in mice, should be aimed to test whether anti-PS antibodies augment lupus-like autoimmunity in genetic strains with a propensity toward disease progression, and conversely whether PS liposomes might also have unexpected therapeutic value. Finally, several enveloped viruses such as Dengue, HIV, and Ebola virus employ apoptotic (PS) mimicry to gain entry to host cells, and blocking PS may also offer therapeutic prospects to block viral entry and immune suppression (101–104).

## CONCLUDING REMARKS

While the link between defective efferocytosis and auto-immune disease and advanced atherosclerosis has been made, and validated in experimental animal models, where and when this circuitry fails in human disease has not been firmly established by genetic causation studies. In recent years, new developments have emerged concerning the mechanisms of PS externalization, and the once seemingly simple paradigm that externalized PS provides a signal for efferocytosis and actively drives a resolution in acute inflammation has been refined by the fact that externalized PS can exist in different functional states. A challenging problem in the field will be to decode the different biological fates of externalized PS, and whether its ability to actively transmit signals is compromised in human autoimmunity. Once the specific conditions can be identified, how exactly PS negatively impinges on chronic inflammation can be elucidated further. These data would be helpful to understand what components of the PS pathways fail during chronic inflammation and autoimmunity.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 30 August 2014; paper pending published: 16 September 2014; accepted: 23 October 2014; published online: 10 November 2014.*
*Citation: Kimani SG, Geng K, Kasikara C, Kumar S, Sriram G, Wu Y and Birge RB (2014) Contribution of defective PS recognition and efferocytosis to chronic inflammation and autoimmunity. Front. Immunol. **5**:566. doi: 10.3389/fimmu.2014.00566*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 12 May 2014
doi: 10.3389/fimmu.2014.00211

# Nuclear receptors and clearance of apoptotic cells: stimulating the macrophage's appetite

***Noelia A-Gonzalez* and Andrés Hidalgo***

*Department of Epidemiology, Atherothrombosis and Imaging, Fundación Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain*

***Edited by:***
*Luis Enrique Munoz, Friedrich-Alexander University Erlangen-Nürnberg, Germany*

***Reviewed by:***
*Patrizia Rovere Querini, Ospedale San Raffaele and Vita-Salute University, Italy*
*Luis Enrique Munoz, Friedrich-Alexander University Erlangen-Nürnberg, Germany*
*Christian Beyer, Friedrich-Alexander University Erlangen-Nuremberg, Germany*

****Correspondence:***
*Noelia A-Gonzalez, Department of Epidemiology, Atherothrombosis and Imaging, Laboratory of Imaging the Cardiovascular Inflammation and the Immune Response, Fundación Centro Nacional de Investigaciones Cardiovasculares (CNIC), Calle Melchor Fernandez Almagro 3, Madrid 28029, Spain*
*e-mail: noelia.alonso@cnic.es*

Clearance of apoptotic cells by macrophages occurs as a coordinated process to ensure tissue homeostasis. Macrophages play a dual role in this process; first, a rapid and efficient phagocytosis of the dying cells is needed to eliminate uncleared corpses that can promote inflammation. Second, after engulfment, macrophages exhibit an anti-inflammatory phenotype, to avoid unwanted immune reactions against cell components. Several nuclear receptors, including liver X receptor and proliferator-activated receptor, have been linked to these two important features of macrophages during apoptotic cell clearance. This review outlines the emerging implications of nuclear receptors in the response of macrophages to cell clearance. These include activation of genes implicated in metabolism, to process the additional cellular content provided by the engulfed cells, as well as inflammatory genes, to maintain apoptotic cell clearance as an "immunologically silent" process. Remarkably, genes encoding receptors for the so-called "eat-me" signals are also regulated by activated nuclear receptors after phagocytosis of apoptotic cells, thus enhancing the efficiency of macrophages to clear dead cells.

**Keywords: macrophages, nuclear receptors, liver X receptors, apoptotic cell clearance, inflammation**

## APOPTOTIC CELL RECOGNITION AND CLEARANCE

Macrophages are professional phagocytes that clear unwanted cells both in the steady-state and during the resolution phase of the immune response. Phagocytosis of apoptotic cells is crucial for development and reproduction. It is also important for the regulation of the immune system because, unlike other phagocytic processes such as phagocytosis of necrotic cells or bacteria, clearance of apoptotic cells does not lead to a pro-inflammatory response in macrophages (1). Apoptotic cell clearance occurs in four steps: sensing of the apoptotic cell, recognition, engulfment of the corpse, and processing of the engulfed material (2). In the last few years, many novel molecules and signaling pathways have been described as key regulators of these steps. In the first step, recognition of the target cell occurs via the so-called "find-me" signals that are released by the apoptotic cell and promote the migration of the phagocyte (3). Examples of these soluble "find-me" signals are the nucleotides ATP and UTP (4), fraktalkine (CX3CL1) (5), and lysophosphatidylcholine (LPC) (6). Apoptotic cells exhibit "eat-me" signals in their surface that are recognized by the phagocyte, either directly or through bridging molecules. The best described "eat-me" signal is phosphatidylserine (PtdSer) exposed in the outer leaflet of the membrane of apoptotic cells (7). Scavenger receptors such as CD36; tyrosine kinases, such as Mertk; or immunoglobulin and mucine domain-containing molecules, such as TIM-4, are membrane receptors that recognize PtdSer (8–10). This recognition may be direct or through soluble factors, such as MFG-E8, Gas6, ProteinS, or the C1q opsonin. The signaling pathways triggered during engulfment then lead to reorganization of the cytoskeleton, and promote internalization of the dying cell (2).

In order to maintain homeostasis, the engulfed material needs to be processed by the phagocyte. When apoptosis occurs, the number of dying cells is typically higher than the number of phagocytes present in the tissue. This disproportion is evident during the resolution phase of inflammation, during the negative selection in the thymus or during germinal center reactions (9, 11–14). However, in all these cases very few if any apoptotic cells can be detected because tissue-resident and recruited macrophages are extremely efficient at clearing up all dying cells, and efficiently processing the extra cargo ingested to prevent the generation of an inflammatory response. This processing entails production of anti-inflammatory cytokines, such as IL-10 and TGF-β1, which are important to initiate the resolution phase or to maintain the process immunologically silent (1, 15, 16). In support of this concept, deficiency in the phagocytosis of apoptotic cells is one of the hallmarks of patients with systemic lupus erythematosus (17). However, the transcriptional regulators of the inflammatory routes triggered by apoptotic cell clearance have only recently begun to be elucidated.

In order to maintain a normal metabolic rate the engulfing phagocyte must process the extra metabolites provided by the ingested apoptotic cells, as excessive metabolite accumulation may

be noxious. Cholesterol efflux is induced in phagocytes by apoptotic cells exposure, and is dependent on phosphatidylserine recognition (18). Expression of genes implicated in cholesterol efflux, such as ATP-binding cassette (ABC) transporter genes, is further up-regulated via activation of nuclear receptors (15, 18–20). This metabolic response is thought to maintain cholesterol levels within the phagocyte. However, macrophages generally ingest more than one apoptotic cell and phagocytosis further enhances recognition and engulfment of apoptotic targets (15, 19). Thus, the extra load of cellular components within the phagocyte might also have energetic benefits for the cell, as it needs energy to continue phagocytizing more cells. Park and collaborators defined an inverse relationship between mitochondrial membrane potential and phagocytosis, in which macrophages with low mitochondrial membrane potential are prone to engulf apoptotic cells. The authors showed that the mitochondrial membrane potential increases in the phagocyte after engulfment of apoptotic cells, to later return to baseline levels. Restoration of baseline potentials is ensured by Ucp2, a mitochondrial membrane protein whose levels also increase after engulfment. Ucp2 therefore acts as a "sensor" for phagocytosis that, by maintaining the mitochondrial membrane potential at basal levels, allows continued phagocytosis (21).

Nuclear receptors are a superfamily of ligand-activated transcription factors implicated in metabolic and inflammatory pathways (22). Their key roles in macrophage biology led us and others to explore their activity in apoptotic cell clearance. This review discusses the importance of nuclear receptors during the phagocytosis of apoptotic cells. We will emphasize how the processing of apoptotic cells, through regulation of metabolic genes and anti-inflammatory pathways, is essential to maintain homeostasis.

## NUCLEAR RECEPTORS AT THE INTERFACE OF METABOLISM AND IMMUNITY

Nuclear receptors share a highly conserved amino-terminal activation domain, a carboxy-terminal ligand binding domain, a zinc-finger DNA-binding domain, and a second activation C-terminal domain (22). Since Mangelsdorf and collaborators first proposed in 1995 a classification of nuclear receptors based on their ligands and DNA-binding modalities (23), several categories have been proposed (24, 25). A simplistic classification of two types of nuclear receptors is described in the Nuclear Receptors Signaling Atlas resource, NURSA (for more detailed information visit NURSA website: www.nursa.org). In the type I category, hormone receptors undergo nuclear translocation upon ligand activation and bind as homodimers to inverted DNA repeat sequences. This category includes estrogen, glucocorticoid, progesterone, mineralocorticoid, and androgen receptors. Type II nuclear receptors are retained in the nucleus and bind as heterodimers with a different nuclear receptor, the retinoid X receptors (RXR), to direct DNA repeats. Thyroid hormone receptor, Liver X Receptors (LXRs), Peroxisome proliferator-activated receptors (PPARs), or Vitamin D receptors (VDRs), among others, fall into this category. Glucocorticoid receptors, LXRs and PPARs have been linked to the phagocytic capacity and phenotypic polarization of macrophages *in vitro* (26–29). However, the mechanism by which gene regulation by these nuclear receptors impacts tissue homeostasis *in vivo* during apoptotic cell clearance is only now starting to be uncovered.

Proliferator-activated receptors are comprised of three isoforms (PPARα, PPARγ, and PPARδ), and are expressed in multiple cell types and tissues. Their endogenous ligands are lipids, such as unsaturated fatty acids, VLDL, and LDL (22). They are essential for fatty acid metabolism by controlling the expression of genes involved in transport, synthesis, activation, and oxidation of fatty acids (30). PPARα activity is mostly restricted to the metabolism of fatty acids, although remarkable immune-regulating properties have been attributed to PPARα due to its capacity to regulate *Cpt1*, a gene involved in T cell function (31).

PPARδ and PPARγ, like other lipid-activated nuclear receptors, are involved in the regulation of inflammatory genes in macrophages. PPARδ is ubiquitously expressed and exhibit pleiotropic functions that range from metabolism, development, and reproduction to inflammation (32). PPARδ represses the expression of inflammatory genes through sequestration of the transcriptional repressor BCL-6 (33). It has been implicated in the phagocytosis of apoptotic cells and prevention of systemic autoimmune diseases (19). Analogous functions in apoptotic cell clearance and autoimmune processes have been described for PPARγ in macrophages (20). Its importance in lipid metabolism is underlined by the variety and function of its target genes, including the scavenger receptor CD36, lipoprotein lipase (LPL), and the nuclear receptor LXRα (34, 35).

LXRα and LXRβ, the two isoforms of LXR, are physiologically activated by oxidized forms of cholesterol. LXRβ is ubiquitously expressed, whereas LXRα is expressed mainly in myeloid cells, intestine, adipose tissue, adrenal glands, and liver. Both isoforms regulate a variety of genes implicated in cholesterol efflux, including the ABC transporters ABCA1 and ABCG1. Accordingly, they have been shown to be important in the prevention of metabolic diseases such as atherosclerosis (22). LXRs can also be pharmacologically activated by potent synthetic agonists that functionally mimic their endogenous ligands.

Elegant studies in the last 10 years have shown that in macrophages previously challenged with inflammatory stimuli, LXRs can act as trans-repressors of pro-inflammatory genes, by binding to other transcription factors and promoting their deactivation (36–39). Thus, like PPARs, LXRs generate cross-talk between inflammation and metabolism. Several studies have now uncovered important roles for these receptors beyond the regulation of inflammatory gene expression and innate immunity. LXRβ has been implicated in the proliferation of T cells, thus influencing adaptive immunity (40). In addition, we have demonstrated that LXRα is essential for the development of two populations of macrophages in the marginal zone of the spleen that are important for immune responses against T cell-independent antigens (41).

The above described pleiotropic functions of PPARs and LXRs position them as excellent candidates to influence macrophage responses during apoptotic cell clearance, in which regulation of metabolic and inflammatory genes is crucial.

## PPARs AND AUTOIMMUNITY

Initial evidence implicating PPARγ in apoptotic cell clearance was obtained in the context of reactive oxygen species

(ROS) production by macrophages (27). In PMA-stimulated macrophages, the production of ROS was attenuated when fed with apoptotic cells. This anti-inflammatory effect was linked to the activity of PPARγ after apoptotic cell clearance. Electrophoretic mobility shift assays revealed transient activation of this nuclear receptor after apoptotic cell recognition (27). Although Mukundan and collaborators later showed that PPARγ transcripts in bone marrow-derived macrophages were not regulated upon phagocytosis (19), several subsequent reports have confirmed a role for PPARγ activation in apoptotic cell clearance (20, 42). In support of the relevance of PPARγ in apoptotic cell phagocytosis by macrophages *in vivo*, mice with conditional deficiency in the receptor in macrophages show a delay in phagocytosis of apoptotic cells and develop autoimmune kidney glomerulopathy (20).

Mice deficient in PPARδ, either globally or restricted to macrophages, also develop a lupus-like autoimmune phenotype characterized by increased levels of autoantibodies in serum and glomerulonephritis (19). This inflammatory phenotype was associated with defective clearance of apoptotic cells by PPARδ-deficient macrophages. Genomic analysis uncovered a number of target genes that were regulated after phagocytosis in a PPARδ-dependent manner. These genes included the C1qb opsonin, which mediates binding of PtdSer to its receptor on the membrane of the phagocyte, and was described as a direct target of PPARδ–RXRα heterodimers. Through this mechanism, phagocytosis is promoted by clearance itself, as double feeding experiments demonstrated that macrophages increased their phagocytic capacity following successive rounds of apoptotic cell feeding (19).

Similarly, RXRα- and PPARγ-deficient macrophages showed impaired apoptotic cell clearance. In addition, engulfment of apoptotic cells failed to down-regulate inflammatory cytokines in LPS-stimulated macrophages derived from RXRα- and PPARγ-deficient mice. As noted above, these mice develop glomerulopathy and proteinuria, both hallmarks of kidney autoimmune disease (20). As with PPARδ, the activity of these nuclear receptors is induced after phagocytosis of apoptotic cells, thereby promoting the transcription of genes encoding membrane receptors and opsonins required for further recognition and engulfment of apoptotic cells. These studies underscore the importance of nuclear receptors in phagocytosis, in part by priming the macrophage for continued engulfment of apoptotic targets.

## LXRs AND APOPTOTIC CELL CLEARANCE: BEYOND MACROPHAGE HOMEOSTASIS

In human macrophages, LXR activation regulates the expression of LXRα (43) and PPARγ (44), thereby creating a positive feedback loop that enhances the phagocytic capacity of macrophages. However, this is not the only role of LXRs in apoptotic cell clearance in human macrophages. Though not a direct target gene of LXRα, Transglutaminase 2 (*Tgm-2*), which encodes a protein-crosslinking enzyme implicated in the phagocytosis of apoptotic cells (45), is regulated in human macrophages after engulfment of apoptotic targets in an LXRα-dependent manner (29). Together with the activity of PPARs during apoptotic cell clearance, these observations establish LXRs and PPARs as molecules that influence the "appetite" of macrophages.

As described above, LXRs are physiologically activated by oxidized forms of cholesterol and are key regulators of cholesterol metabolism by controlling the expression of genes responsible for cholesterol efflux, such as ABCA1. This raises the question of what is the significance of LXR activation during apoptotic cell clearance. When a macrophage ingests an apoptotic cell, the amount of cellular content within the macrophage significantly increases, and the extra cellular material has to be processed. A potential solution to this dilemma is the up-regulated expression of a gene responsible for cholesterol efflux, *Abca1*, upon engulfment of apoptotic cells (18, 46). LXR activation appears to be required for this upregulation of *Abca1* because, in peritoneal macrophages obtained from mice deficient in both LXR isoforms (LXRαβ−/−), changes in *Abca1* mRNA expression were blunted after apoptotic cell clearance when compared to control macrophages (46). Moreover, Kiss and collaborators demonstrated that *Abca1* expression and cholesterol efflux were induced upon PtdSer recognition by the macrophage, implying that engulfment is dispensable for LXR activation. In support of a role for nuclear receptors in cholesterol processing, LXRs and PPARγ antagonists inhibited upregulation of *Abca1* and cholesterol efflux mediated by apoptotic cell clearance (18).

At the time of these studies, LXR activation had been exclusively linked to the metabolic response of the phagocyte during apoptotic cell clearance. Using LXR knock-out mice we observed an impaired phagocytic capacity in LXR-deficient macrophages both *in vivo* and *in vitro*, and this impairment was associated to the development of autoimmunity in these mice (15). Apoptotic cells promote the expression of a number of genes in macrophages after clearance. Some of these genes are regulated in an LXR-dependent manner, such as genes implicated in cholesterol metabolism, glucose transport, and other genes identified as LXR target genes in other studies. Similarly, the expression of *Mertk*, a membrane receptor for apoptotic cells, was also up-regulated by LXRs during phagocytosis or after activation with synthetic LXR ligands, and was identified in these studies as a novel direct target of LXR (15). Together, the responses triggered by LXR activation contribute to enhancing recognition and further engagement of apoptotic targets as evidenced by the observation that the phagocytic capacity in LXRαβ−/− macrophages does not increase after several rounds of apoptotic cell feeding. Notably, by modulating the expression of inflammation-related genes, LXR also participates in the polarization of the macrophage toward an anti-inflammatory phenotype after engulfment of dying cells. This activity essentially contributes to avoidance of unwanted inflammation, which is illustrated by the lupus-like autoimmune disease developed by LXRαβ−/− mice as they age (15).

Liver X receptors nuclear receptors have more recently emerged as regulators of neutrophil homeostasis (47, 48). Due to their short half-life (estimated in 12.5 h in mice), neutrophils must be efficiently cleared on a daily basis. LXR-deficient mice display neutrophilia in blood and accumulation of neutrophils in the spleen and liver, a phenotype that was accounted for by the impaired capacity of LXR-deficient macrophages to engulf apoptotic neutrophils. Production of IL-23 by macrophages and dendritic cells is a critical signal that controls the levels of neutrophils in blood by acting upstream of IL-17 and G-CSF (49). Importantly,

activation of LXRs upon neutrophil engulfment strongly represses IL-23 transcription (47). Extending these studies, we have recently shown that clearance of aged neutrophils in the bone marrow modulates the size and activity of the hematopoietic niche through LXR activation (48). We found that neutrophils cleared from blood enter the bone marrow and are engulfed by macrophages, leading to reductions in the number of niche cells and mobilization of hematopoietic progenitors into the bloodstream. In addition, the transcript levels of LXR target genes in the bone marrow spontaneously increase at the time when neutrophils are cleared in this organ, and mice in which macrophages have been eliminated lacked the normal oscillations in *Abca1* expression. Further, regulation of niche cells and progenitor release are impaired in LXR-deficient mice. Together, these findings uncovered new functions for the homeostatic clearance of dying cells in regulating hematopoietic niches in the bone marrow, and a central role for LXR receptors in coordinating these functions (48).

These recent advances in the field provide examples of the multitude of processes and tissues that are likely regulated by the clearance of apoptotic cells by macrophages, and by the receptors involved in this fundamental process.

## FUTURE DIRECTIONS

Liver X receptors and PPARs are now recognized regulators of the anti-inflammatory response in macrophages after clearance of apoptotic cells. Moreover, these receptors are key players in the recognition and engagement of apoptotic cells by further enhancing phagocytosis through transcriptional regulation of various receptors and bridging molecules. The exact pathways by which LXRs and PPARs are activated during the phagocytosis of apoptotic cells remain an open question in the field (**Figure 1**). We and others postulated that lipids derived from the engulfed cell might provide ligands for PPARs and LXRs, as demonstrated by the lack of LXR activation when macrophages are fed with sterol-free apoptotic thymocytes (15). Because recognition of PtdSer by macrophages is sufficient to activate an LXR-dependent metabolic program without engulfment (18), additional routes of recognition and engulfment can activate these nuclear receptors.

These novel roles of lipid-activated nuclear receptors in phagocytosis of apoptotic cells raise an interesting issue regarding cell metabolism and bioenergetics. The enhancement of phagocytosis of apoptotic cells mediated by nuclear receptors, might respond to a necessity of generating more energy to continue phagocytizing. Mitochondria provide the majority of the energy supply by oxidative phosphorylation in the respiratory chain. In fact, macrophages with low mitochondrial membrane potential are more prone to phagocyte apoptotic cells (21). Whether nuclear receptors and mitochondria cross-talk during apoptotic cell clearance to enhance phagocytosis arises as an interesting possibility. Supporting this idea, the activity of several nuclear receptors have been defined

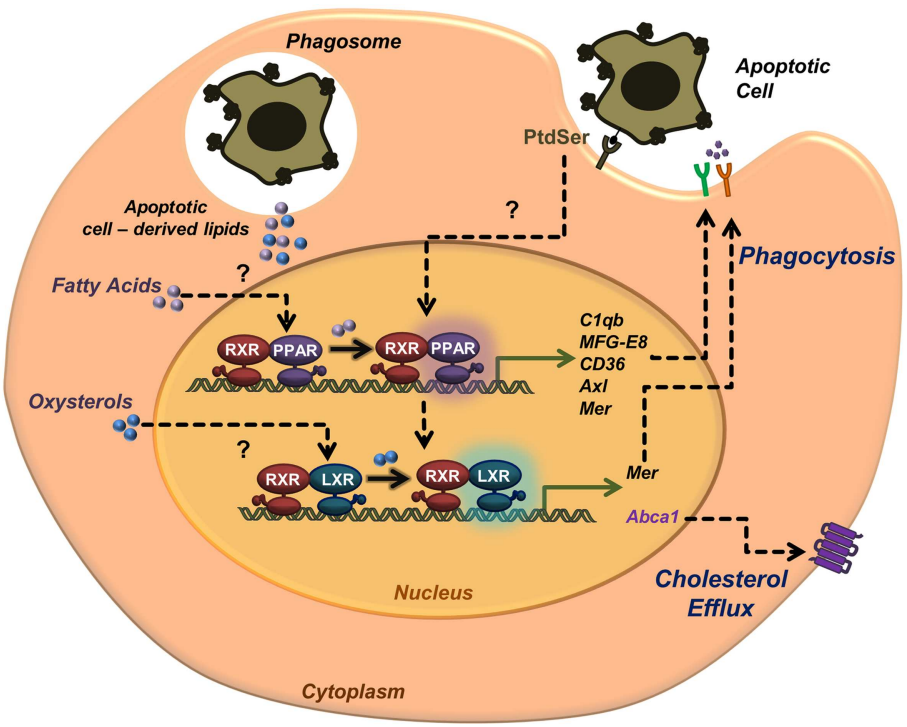


**FIGURE 1 | Activation of nuclear receptors in phagocytes during apoptotic cell clearance**. Apoptotic cell recognition and engulfment promote the transcriptional activity of nuclear receptors LXRs and PPARs. Recognition of phosphatidylserine in the outer leaflet membrane of the apoptotic cell leads to transcriptional activation of ABCA1 and cholesterol efflux. Nuclear receptor activation upon apoptotic cell phagocytosis also leads to upregulation of phagocytic receptors (e.g., Mer, CD36, and Axl) and opsonins (e.g., MFG-E8 and C1qb). Lipids derived from the engulfed apoptotic cells may also serve as source of endogenous ligands to activate PPARs (fatty acids) and LXRs (oxysterols).

in mitochondria, regulating gene expression, coordinated with nuclear gene expression, in situations of high energy demand (50). For example, PPARγ co-activator 1α, PGC-1α, collaborates with PPARs to regulate expression of mitochondrial enzymes involved in fatty acid transport and oxidation (51). However, the specific role of nuclear receptors in mitochondrial metabolism during apoptotic cell clearance remains unclear.

An important outcome of this research topic will be the potential therapeutic implications of apoptotic cell clearance in a wide range of inflammatory and metabolic diseases. It has been shown that enhancing engulfment of apoptotic neutrophils *in situ* accelerates the resolution of bacterial infection and lung inflammation (52–54). However, the exogenous administration of apoptotic cells could also lead to autoimmunity, so the therapeutic approaches need to be finely controlled to avoid deleterious effects (55). Targeting nuclear receptors by activation through synthetic ligands, have been proven to ameliorate inflammation in mouse models of autoimmunity (15) and atherosclerosis (56). Though some PPAR agonists have already been approved for clinical use to treat metabolic diseases, a better understanding of nuclear receptor activation during apoptotic cell clearance may pave the way for the development of novel treatments for infectious, inflammatory, and metabolic diseases.

## ACKNOWLEDGMENTS

Both authors contributed to the writing and editing of this review. We thank Linnea A. Weiss, María Casanova-Acebes, and Jose Angel Nicolas for critical comments on the manuscript. This mini-review was supported by grant SAF2012-31142 from the Spanish Ministry of Economy and Competitivity (MINECO) to Andrés Hidalgo, the Juan de la Cierva program (JCI-2012-12659) from MINECO to Noelia A-Gonzalez, and the FP7-People-IRG Program (246655) to Andrés Hidalgo. The Centro Nacional de Investigaciones Cardiovasculares is supported by MINECO and the Pro-CNIC Foundation.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 25 February 2014; accepted: 28 April 2014; published online: 12 May 2014.*
*Citation: A-Gonzalez N and Hidalgo A (2014) Nuclear receptors and clearance of apoptotic cells: stimulating the macrophage's appetite. Front. Immunol. **5**:211. doi: 10.3389/fimmu.2014.00211*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

OPINION ARTICLE
published: 30 July 2014
doi: 10.3389/fimmu.2014.00365

# Clearance of apoptotic bodies, NETs, and biofilm DNA: implications for autoimmunity

***Marko Radic****

*Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA*
**Correspondence: mradic@uthsc.edu*

***Edited by:***
*Martin Herrmann, Universitätsklinikum Erlangen, Germany*

***Reviewed by:***
*Johan Van Der Vlag, Radboud University Nijmegen Medical Centre, Netherlands*
*Christine Schorn, University Hospital Erlangen-Nuremberg, Germany*

**Keywords: apoptosis, NETosis, clearance, autoimmunity, autoantibodies, lupus**

## INTRODUCTION

Apoptosis and NETosis, two important pathways of programed cell death, differ in their morphologic features and their effects on the immune system. In apoptosis, nuclear chromatin compacts as it is packaged into nuclear fragments and apoptotic blebs (1), and uptake of apoptotic cells by phagocytes generally suppresses the immune response (2). In NETosis, named after neutrophil extracellular traps (NETs), nuclear chromatin relaxes and forms a fibrous meshwork upon release from the cell (3). In general, NETosis is induced by infection, inflammation, or trauma and represents a mechanism of innate immune activation (4). Neutrophils, the most abundant type of white blood cells, migrate toward a stimulus in coordinated fashion, and NETs may synchronize such neutrophil swarms (5). Despite the structural and functional differences between apoptosis and NETosis, significant aspects of their clearance pathways likely overlap, as specific serum proteins participate in the recognition and uptake of remnants from either cell death pathway. *In vivo*, it is likely that both cell death pathways are concurrently present and that apoptotic bodies and NETs entangle (6). Yet, a third type of DNA may intertwine with DNA from apoptotic and NETotic cells, as certain bacteria and fungi release extracellular DNA that is used to construct biofilms (7). How apoptotic bodies, NETs, and biofilm DNA (**Figure 1**) are safely cleared is of great interest, because incomplete clearance leads to systemic inflammation and autoantibody production.

## SYSTEMIC AUTOIMMUNE DISEASES AND AUTOANTIBODIES TO NUCLEAR ANTIGENS

Molecular structures associated with dying cells are targets of autoantibodies in autoimmune diseases such as systemic lupus (SLE) (8), antiphospholipid syndrome (APS) (9), as well as other musculoskeletal/rheumatoid disorders (10). The resulting autoreactivities are idiosyncratic for each condition and thus are useful for clinical diagnosis. However, the antigens recognized by the autoantibodies are also involved in pathogenesis, as they accumulate at the sites of tissue damage and contribute to immune complex deposition (11). Tissue damage may worsen in the absence of serum nucleases such as DNAse I (12). Furthermore, the interactions between dying cells and the adaptive immune system strengthen over time, as somatic mutations and antigen selection optimize antibodies for improved binding (13). In SLE, antibodies to nuclear or plasma membrane antigens arise in the course of disease (14, 15). These antibodies avidly bind to apoptotic cells (16). Classical studies recognized that apoptotic cells are far better substrates for autoantibody binding than viable cells (17). However, monoclonal antibodies from mouse lupus models that bind to apoptotic blebs (16) also tightly bind to NETs released in response to bacterial pathogens (18). Our laboratory showed that NETotic cells provide suitable targets for autoantibodies from diverse human autoimmune disorders (19). Whether apoptotic or NETotic cell death, or both, provide antigens that induce autoantibody production is

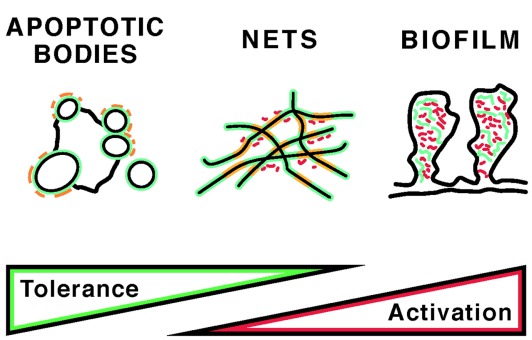


**FIGURE 1 | Self and foreign antigens that may induce autoantibodies in autoimmunity**. The potential contribution of apoptotic bodies, NETs, and bacterial biofilms to immune tolerance versus stimulation is indicated. The distribution and content of self (green) and foreign (red) antigens is diagrammed. In apoptotic bodies, "foreign" structures may include post-translational modifications that are present only during late stages of apoptosis (orange). NETs, in addition to modified chromatin (orange), may also contain bacterial adjuvants, whereas biofilms may incorporate host DNA. Short red rods indicate bacteria in NETs and biofilms. For details, see text.

essential information for understanding the etiopathogenesis of autoimmune diseases (20).

## APOPTOTIC AND NETotic CELL DEATH

Apoptosis is characterized by dramatic morphologic changes that are orchestrated by a family of specific proteases called caspases (21). The chromatin in the nucleus condenses tightly despite the fact that caspase-activated DNAse cleaves certain regions of genomic DNA to produce an oligonucleosome "ladder" (22). Curiously, the diameter (and thus the permeability) of nuclear pores transiently increases during this stage of apoptosis (23), and oligonucleosomes pass through the pores into the cytoplasm (16). The chromatin fragments associate with the outer nuclear envelope, the nucleus breaks up, and nuclear fragments migrate toward the cellular plasma membrane. These nuclear fragments form "blebs" at the cell surface, which are characteristic protrusions that give apoptotic cells their typical "grape cluster" appearance. Blebs display DNA, chromatin, and ribonucleoproteins at the cell surface (16, 24) such that these autoantigens become accessible to antibodies and pattern recognition receptors.

An alternative form of cell death was discovered by Brinkmann et al. (18). These authors reported that, upon exposure to bacteria, LPS, or PMA, neutrophils dissolve nuclear and cytoplasmic granule membranes, relax nuclear chromatin, associate the chromatin with granule components such as myeloperoxidase or elastase, and release the relaxed chromatin across the plasma membrane (4). The chromatin appears as disorganized fibers that spread widely to form an extracellular network. The authors named the fibers "NETs" because this chromatin could immobilize or "trap" bacteria. Mouse anti-chromatin antibodies were used to demonstrate that the NETs consisted of DNA and histones. These results immediately suggested that a tangle of bacteria and nuclear chromatin should be viewed as a "dangerous liaison" between lupus autoantigens and bacterial adjuvants that, by acting as a molecular complex, could trigger an adaptive immune response (25).

Follow-up studies revealed that NETs are not always an impediment to microbes. Proliferation assays identified certain species of bacteria that are resistant to any bactericidal effects of the released neutrophil chromatin (26), even though NETs organize bactericidal granule contents such as peroxidase and serine proteases (27), and even though histones also exhibit bactericidal activity (28). In fact, NET chromatin has found a novel use for certain bacteria that can incorporate NET chromatin into their extracellular matrix (29, 30). Such biofilms protect the microbes from physiological and pharmaceutical antibiotics and help to colonize various host tissues (7). DNA gives biofilms their structural integrity because nuclease treatment efficiently dissolves biofilms (31). The biofilms can also incorporate microbial DNA, as particular bacteria and fungi have mechanisms to release sections of genomic DNA for use in forming biofilms. Such DNA could be of particular significance in inducing anti-DNA responses because bacterial DNA has hypomethylated CpG motifs that directly stimulate toll-like receptors (32) and other DNA receptors (33) in B cells and other antigen-presenting cells.

## EVIDENCE FOR APOPTOSIS AND NETosis IN THE INDUCTION OF AUTOIMMUNITY

Evidence supporting apoptotic cells as the source of autoantigens that induce and promote the development of autoimmunity derives from a close inspection of autoantibody specificities. The observation that lupus serum IgG bind to apoptotic cells (17) initiated an active area of research. Because apoptotic cells externalize phosphatidylserine at the cell surface, binding of serum factors or lupus antibodies to phosphatidylserine could interfere with clearance in a way that would alter recognition of apoptotic cells and potentially induce disease. This view is consistent with genetic defects in cell clearance that in many instances recreate the full set of lupus manifestations (8).

Completion of the apoptotic program without adequate clearance may lead to the exposure of highly modified autoantigens (34). Autoantibodies to apoptotic cells may be induced by unique antigenic structures that are produced by enzymatic reactions in apoptotic cells. Granzyme B activation in apoptosis was identified as one possible mechanism whereby apoptosis generates novel self antigens that stimulate autoantibody binding (35). Importantly, characteristic post-translational modifications (PTM) of histones are induced during apoptosis. These include the acetylation of lysine 12 in the H2B core histone, a PTM that was shown to enhance the binding of lupus autoantibodies (36). However, lysine 12 acetylation also occurs in NETosis, and tri-acetylated histone H4, a specific target of the KM-2 murine lupus autoantibody, is more abundant in NETs from SLE patients than in controls (37). Therefore, antibody reactivity against any single histone PTM may not unambiguously establish which biological process supplies nuclear antigens in autoimmunity (38).

The generation of apoptotic cells during development and under conditions of rapid cell turnover, such as exist physiologically in primary lymphoid organs, suggests that apoptotic lymphocytes provide a steady supply of tolerogenic autoantigens (39). The idea that apoptosis provides self antigens that maintain tolerance is supported by immune suppression following injection of apoptotic cells (40). Immune suppression by apoptotic cells can also be recreated *in vitro* (41) and can be converted to immune activation by opsonization of apoptotic cells with antibodies (42). On balance, NETosis is a more likely alternative source of autoantigens that stimulate autoreactive B cells. This follows directly from the observation that, in autoimmunity, autoantibodies arise to various known NET components (43, 44). These include the proteases cathepsin G, proteinase 3, and elastase, as well as granule peptides, including LL37 and other defensins that have bactericidal properties.

Detailed analysis revealed that neutrophils from autoimmune patients are more prone to NETosis than controls and that NETosis is associated with particular autoantigen modifications (45, 46). Such autoantigen PTM may arise through reactive oxygen species liberated in NETosis or through enzymes that are activated during the progression of NETosis. Amino acids such as tryptophan and tyrosine are modified by oxidation or reactions with hypochlorous acid and peroxynitrite (47). NETosis also activates peptidylarginine deiminases (PADs), enzymes that convert arginine residues in proteins to citrulline residues. Our laboratory was

first to link deimination (also known as "citrullination") of nucleohistones to steps that are set in motion during NETosis (25). Importantly, we also showed that histone deimination is independent of caspase activity and that induction of apoptosis prevents PAD activation. Thus, deimination of histones clearly distinguishes NETosis from apoptosis.

In subsequent studies, we showed that citrullinated histones, including core and linker histones, are recognized in preference over non-modified histones by antibodies from patients with various autoimmune diseases, including SLE and Felty's syndrome, a more severe form of rheumatoid arthritis (10). In confirming our results, others have shown that autoantibodies to deiminated histones are remarkably useful in the diagnosis of rheumatoid arthritis (48). In earlier studies, it was reported that citrullinated proteins are frequently targets of IgG antibodies from patients with arthritis (49), and antibodies to citrullinated antigens have been a focus of a growing number of research studies (50, 51). These observations represent a solid link between NETosis and the induction of disease-specific autoantibodies.

## CLEARANCE MECHANISMS

Clearance of apoptotic cells has been a focus of research for more than two decades (52), and a bewildering complexity of pathways has emerged (53). Different cell types participate in the uptake of apoptotic cells, the cells employ different combinations of receptors, and clearance may be enhanced or suppressed by various plasma proteins. Soluble plasma proteins that participate in apoptotic cell clearance include members of the pentraxin (54) and collectin families (55), the complement protein C1q (56), and milk fat globule epidermal growth factor 8 (MFG-E8) (57). An important "eat-me" signal is generated by the endoplasmic reticulum chaperone calreticulin. Apoptotic cells release calreticulin from the endoplasmic reticulum into the cytoplasm (58). The cytoplasmic calreticulin binds to phosphatidylserine in the inner leaflet of the plasma membrane from where it is externalized as the plasma membrane loses its asymmetry. At the cell surface, calreticulin combines with C1q and binds CD91 on the surface of the macrophage, leading to the phagocytosis of the apoptotic cell (59). Other receptors for uptake of apoptotic cells include SCARF1, a highly conserved receptor for C1q (60), and the integrin $\beta_V\alpha_5$, a receptor for MFG-E8 (61). The importance of C1q, MFG-E8, and SCARF1 for tissue homeostasis is emphasized by the fact that mice deficient for any of these molecules show a reduced capacity for apoptotic cell clearance and exhibit a concomitant induction of autoantibodies (60, 62, 63). In SLE, altered levels of MFG-E8 in the serum and impaired C1q recognition of apoptotic cells correlate with the severity of disease manifestations (64, 65).

Additional receptors for the recognition and clearance of apoptotic cells are the Mer, Axl, and Tyro3 receptor tyrosine kinases (66). Mice deficient in any of these receptors manifest symptoms of autoimmune disease (67), and patients show altered serum levels of Mer family ligands GAS6 and protein S (68). Whereas Axl determines apoptotic cell clearance by dendritic cells (69), Mer is induced by C1q and serves to enhance apoptotic cell uptake by macrophage (70). It is important to note that several of these receptor–ligand systems are not specific for apoptotic cells but instead participate in the clearance of infectious microbes such as bacteria, fungi, and viruses (53). Possibly, some of these clearance pathways also serve to eliminate other cellular remnants.

Little is known about the clearance of NETotic cells, although a systematic analysis of the relevant mechanisms for NET clearance is urgently needed. Good starting points would be proteins and receptors that bind DNA or chromatin and that participate in the clearance of apoptotic cells. For example, several pentraxins (71) and collectins (55) bind to nucleic acids and chromatin, and calreticulin exhibits high affinity for chromatin and nucleosomes (72). It is likely that these proteins and receptors also bind NETs, although NETs are not efficiently recognized by the pentraxin C-reactive protein, or the complement protein C3b (73). In contrast, C1q binds NETs and activates the complement cascade (74, 75). The search for additional factors that regulate NET clearance is timely because NETosis has been linked to atherosclerosis (76), small vessel vasculitis (77), deep vein thrombosis (78), and various autoimmune conditions (79). Conversely, autoimmune diseases show an aberrant persistence of NETs, and NET clearance is impaired in APS (80), SLE (81), and gout (82). A better knowledge of NET clearance is expected to lead to new treatments for autoimmune diseases, as inhibitors of PAD4 show promise in various animal models of autoimmune disorders (83–86).

## ACKNOWLEDGMENTS

Thanks to Tim Higgins, Senior Scientific Illustrator for the design of (**Figure 1**). Research in the Radic lab is supported by the ORR Fund of Memphis, TN, USA.

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**Conflict of Interest Statement:** The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 20 June 2014; accepted: 15 July 2014; published online: 30 July 2014.*
*Citation: Radic M (2014) Clearance of apoptotic bodies, NETs, and biofilm DNA: implications for autoimmunity. Front. Immunol. **5**:365. doi: 10.3389/fimmu.2014.00365*
*This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 09 April 2014
doi: 10.3389/fimmu.2014.00164

# Breaking immunological tolerance in systemic lupus erythematosus

***Elmar Pieterse and Johan van der Vlag****

*Department of Nephrology, Radboud University Medical Center, Nijmegen, Netherlands*

***Edited by:***
*Martin Herrmann, Universitätsklinikum Erlangen, Germany*

***Reviewed by:***
*Angelo A. Manfredi, Vita Salute San Raffaele University, Italy*
*Luis Enrique Munoz, Friedrich-Alexander University, Germany*

****Correspondence:***
*Johan van der Vlag, Department of Nephrology, Radboud University Medical Center, 480, P.O. Box 9101, Nijmegen 6500 HB, Netherlands*
*e-mail: johan.vandervlag@radboudumc.nl*

Systemic lupus erythematosus (SLE) is a fairly heterogeneous autoimmune disease of unknown etiology that mainly affects women in the childbearing age. SLE is a prototype type III hypersensitivity reaction in which immune complex depositions cause inflammation and tissue damage in multiple organs. Two distinct cell death pathways, apoptosis and NETosis, gained a great deal of interest among scientists, since both processes seem to be deregulated in SLE. There is growing evidence that histone modifications induced by these cell death pathways exert a central role in the induction of autoimmunity. In the current review, we discuss how abnormalities in apoptosis, NETosis, and histone modifications may lead to a break of immunological tolerance in SLE.

**Keywords: systemic lupus erythematosus, apoptosis, neutrophil, NETosis, histone modifications**

## INTRODUCTION

Autoimmune disorders are disturbances of the immune system that arise when the immune system responds to self. Immunological tolerance to self relies on the immune system to discriminate self from non-self. Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder, in which primarily nuclear constituents, i.e., DNA, histones, and ribonucleoproteins, are targeted (1). In a substantial subset of SLE patients, autoantibodies also target proteins from the cytoplasm of neutrophils (2). Since these nuclear self-antigens are normally shielded from the extracellular space by the nuclear membrane and the cell membrane, the key question is how these nuclear self-antigens are released and become exposed to the immune system. Anno 2014 abnormalities in two pathways of cell death, apoptosis and NETosis, are recognized as central processes that provide nuclear autoantigens and drive the autoimmune response in SLE (3).

## TWO SOURCES OF AUTOANTIGENS IN SLE

For over 20 years, apoptosis has been considered as the major source of autoantigens in SLE (4). Apoptosis is a highly organized and immunologically silent cell death pathway that plays an important role in tissue homeostasis. In processes characterized by a high-rate of tissue turnover, such as embryogenesis in human development, apoptosis is a crucial mechanism that allows tissues to remodel without triggering inflammation. Many cellular pathways and signals can activate proteolytic caspases to break down the cell in a strictly controlled and fine-tuned manner that distinguishes apoptosis from any other form of cell death. Apoptosis can be induced actively through ligation of cell surface receptors such as Fas or TNFR or passively through lack of essential survival signals. Apoptotic cells undergo a series of distinct morphological changes, including cytoskeletal disruption, cell shrinkage, DNA fragmentation, and plasma membrane blebbing (5). It has been shown that many of the nuclear autoantigens targeted in SLE are concentrated within apoptotic blebs (6, 7).

A specialized form of neutrophil cell death, termed NETosis, has been described a decade ago (8). NETosis has been linked to SLE as an additional source of autoantigens (9). During NETosis, neutrophils extrude fibrillary networks composed of DNA, citrullinated histones, and granule peptides such as neutrophil elastase, myeloperoxidase, and cathepsin G. These structures are termed neutrophil extracellular traps (NETs) and serve to entrap and dismantle not only extracellular bacteria, but also viruses, fungi, and parasites (10–12). In addition to pathogens, sterile inflammatory mediators such as monosodium urate (MSU) crystals, IL-8, IL-1β, platelet-activating factor (PAF), and TNF-α have been reported to induce NETosis (13). NETosis requires a very rapid disintegration of the nuclear envelope, translocation of granule peptides to the nucleus, PAD4-mediated citrullination of the chromatin, binding of granule peptides to citrullinated chromatin, and finally rupture of the plasma membrane (14). Where apoptosis is organized and planned, NETosis seems much faster and less well-coordinated.

## INCREASED CELL DEATH

MRL/lpr mice, the most commonly studied murine model for lupus-like disease, develop an autoimmune disease that reflects pathologies of human SLE, including lymph node enlargement, increased IgG levels, antinuclear autoantibody production, glomerulonephritis, proteinuria, and development of skin lesions (15). MRL/lpr mice express a defective form of the Fas receptor that under physiological conditions stimulates cells to undergo apoptosis. This initially led to the belief that SLE patients have a similar defect in Fas-mediated apoptosis that underlies the failure of self-tolerance. However, it has become clear that SLE is quite

the opposite from being a disease with impaired apoptosis. Substantially, evidence correlates increased lymphocyte, neutrophil, macrophage, and monocyte apoptosis directly to SLE disease activity (16–19). It has been demonstrated that SLE serum has a strong apoptosis-inducing capacity in macrophages, monocytes, and lymphocytes from healthy donors (20). In addition, it has been reported that autoreactive T cells show an increased expression of the apoptotic ligands TRAIL, TWEAK, and FasL that directly mediate the apoptosis of monocytes (21).

Since various bactericidal NET proteins were found to be present at much higher levels in blood from patients with SLE compared to healthy donor blood, enhanced NETosis is also implicated in the genesis and/or amplification of the autoimmune response in SLE (22–24). The pro-inflammatory cytokines IL-17A, TNF-α, IL-1β, and IL-8 have all been reported to induce NETosis, suggesting that NETs are extensively formed in an inflammatory environment such as in SLE (25–27). Elevated levels of interferon-alpha (IFN-α), a cytokine that under physiological conditions is important for antiviral responses and immune activation, have been correlated with disease activity in SLE (28). Recent evidence indicates that NETosis is enhanced in SLE due to priming of neutrophils by IFN-α (29). Certain autoantibodies that are highly present in the majority of SLE patients, such as anti-LL-37, anti-HNP, and anti-RNP, can also stimulate neutrophils to produce NETs in a FcγRIIa-dependent manner (30–32). Furthermore, a distinct subset of neutrophils in SLE patients, so called low-density granulocytes (LDGs), have been described with an increased NET-releasing capacity due to their ability to synthesize IFN-α in an autocrine manner (33).

Taken together, it is presumed that both apoptosis and NETosis occur excessively in patients with SLE, which results in an increased load of nuclear autoantigens. However, can this excessive release of nuclear autoantigens explain the break of immunological tolerance to nuclear antigens in SLE? Apoptosis and NETosis are physiological forms of cell death. In our daily battle against pathogens, we release NETs and between 50 and 70 billion cells die daily due to apoptosis. Humans evolved redundant mechanisms to clear apoptotic material and NETs. This clearance is usually accompanied by secretion of anti-inflammatory cytokines (34). Multiple groups tried to immunize mice with apoptotic cells/blebs or NETs, but this never lead to considerable immune activation (35, 36). Therefore, it can be concluded that increased apoptosis or NETosis on its own is not sufficient to break immunological tolerance to nuclear autoantigens in SLE, and that additional factors are required to turn apoptotic material or NETs into dangerous triggers of autoimmunity.

## CLEARANCE DEFICIENCY IN SLE

In 1980, it was for the first time described that macrophages from SLE patients show an impaired phagocytic activity for yeast (37). Later, it was described that the phagocytosis of autologous apoptotic material by monocyte-derived macrophages is also disturbed in about 50% of the SLE patients (38). This finding was confirmed by other groups (18, 39). Interestingly, macrophages differentiated from CD34 positive stem cells of SLE patients show a different morphology than those generated from healthy donors; they are relatively small and poorly ingest apoptotic material (40). Furthermore, the amount of tingible body macrophages (TBMs) found in germinal centers appears to be strongly reduced in SLE patients (41). Apoptotic material in germinal centers is normally internalized by TBMs. In SLE patients with a reduced number of TBMs, apoptotic material was observed to be directly associated with the surface of follicular dendritic cells (FDCs), which may provide survival signals for autoreactive B cells. Monocytes and granulocytes from SLE patients display a reduced phagocytic activity as well (42), which may be explained by the relative low expression of the phagocytic receptor CD44 on both cell types (43).

In addition to well-functioning healthy phagocytes, serum proteins have an important impact on the clearance of apoptotic cells as well. Adequate removal requires a clear recognition of apoptotic cells, which is, in addition to the exposure of phosphatidylserine on the outer leaflet of its plasma membrane, strongly dependent on opsonizing proteins such as immunoglobulin M (IgM), mannose-binding lectin (MBL), serum amyloid P (SAP), C-reactive protein (CRP), and C1q. Numerous studies ascribe a role for these opsonins in the defective clearance of apoptotic material in SLE. It has been shown that decreased IgM levels and increased MBL levels correlate with an increased disease activity in SLE (44, 45), that administration of SAP and CRP significantly delays disease onset and alleviates disease symptoms (46, 47) and that C1q-deficient mice rapidly develop autoantibodies against nuclear autoantigens (48). Polymorphisms at the loci of the genes encoding these opsonins as well as the formation of autoantibodies against these opsonins are considered to be the underlying cause for their absence or defective functioning in SLE (49–52). Lastly, autoantibodies against pentraxin-related protein PTX3, a cytokine-induced protein that is homologous to CRPs and SAPs, appear to be frequently present in SLE patients as well (53). In contrast to anti-CRP or anti-SAP autoantibodies, anti-PTX3 autoantibodies are not associated with disease activity but with the absence of glomerulonephritis and antiphospholipid antibodies. The authors of this article suggest that PTX3 inhibits the clearance of apoptotic material, which is counteracted by the autoantibodies directed against them.

There is also evidence for an impaired clearance of NETs. Deoxyribonuclease I (DNase I) plays a crucial role in the degradation of NETs, which is not surprising since the backbone of NETs is composed of nuclear DNA. The relevance of proper DNase I activity is reflected by the fact that DNase I-deficient mice develop a syndrome that closely resembles to SLE (54). A considerable number of SLE patients display a reduced DNase I activity (55). These patients develop relative high titers of anti-dsDNA autoantibodies and suffer from more severe symptoms. Low DNase I activity in SLE may have a genetic cause (56) but can also be the result of inhibitory molecules or anti-DNase I autoantibodies. In a Taiwanese cohort, 62% of the SLE patients appeared to be positive for anti-DNase I autoantibodies compared to only 8% of normal controls (57). Comparing sera from healthy donors and patients with SLE in their capability of degrading NETs *in vitro* revealed that 98.1% of the healthy donor sera degraded NETs normally, whereas a significant percentage of the SLE sera did not (36.1%) (58). Interestingly, those patients who could not degrade NETs developed lupus nephritis significantly more frequently than those who could degrade NETs. Depleting autoantibodies from

SLE sera considerably enhanced NET degradation, suggesting that NET-bound autoantibodies inhibit NET degradation, most likely by preventing the access for DNase I to the NET. In addition, it was shown that NET-bound C1q also inhibits NET degradation (59) via the same mechanism. However, C1q seems to be a double-edged sword in the removal of NETs: both recombinant C1q and endogenous C1q derived from human serum were found to opsonize NETs for their immunologically silent clearance by macrophages (60). Lastly, it has been shown that the antimicrobial peptides LL-37 and HMGB1 prevent NET degradation (61). This latter observation is interesting, since it has been described that these antimicrobial peptides are highly present in NETs from SLE patients but not in those from healthy donors (32).

The contribution of intrinsic phagocyte defects and absent/deficient serum factors to the impaired clearance of apoptotic material and/or NETs in SLE seems clear. However, it is believed that there are numerous additional factors and pathways that could play a role in the complex pathogenesis of SLE. Although many different pathways may be deregulated, that all lead to a comparable SLE phenotype, it is assumed that accumulation of apoptotic material and NETs in tissues is the common denominator between all patients with SLE (42). Nevertheless, the question remains how such an accumulation of apoptotic material and NETs can break immunological tolerance. After all, it involves an accumulation of endogenous material that is not supposed to elicit an autoimmune response. An important hypothesis that gains growing support states that biochemical reactions, for example cleavage by caspases or protein modifications by protein modifying enzymes, lead to enrichment of protein modifications in not efficiently cleared NETs or apoptotic cells (62). Certain (combinations of) protein modifications may give rise to proteins with neoantigens that behave as danger signals as well and thereby are no longer perceived as endogenous and therefore have the ability to initiate an autoimmune response. Neoantigens/danger signals in NETs are directly exposed to the immune system, but apoptotic blebs require to undergo secondary necrosis, a late apoptotic stage characterized by loss of membrane integrity and leakage of cellular constituents, for exposure of these neoantigens/danger signals to the immune system (63). Neoantigens/danger signals may be ingested, digested, and presented in an immunogenic way by antigen-presenting cells in MHC class II to autoreactive CD4+ T cells, who subsequently instruct autoreactive B cells to produce autoantibodies against them. Alternatively, extracellular autoantigens may be presented in MHC class I, via the mechanism of cross-presentation, to autoreactive CD8+ T cells (64). The concept of epitope spreading may ultimately lead to the wide arsenal of autoantibodies that are characteristic for SLE. An important group of proteins in which post-translational modifications (PTMs) seem to play an important role in (the induction of) autoimmune responses in SLE are histones (65).

## HISTONE MODIFICATIONS RELATED TO SLE

Histones are a group of chromatin proteins that are abundantly present in apoptotic blebs as well as in NETs. Anti-histone autoantibodies are frequently found in SLE and are disease-specific (66). Under physiological conditions, histones play a critical role in the packaging of nuclear DNA. Eukaryotic cells possess five major families of histones: H1/H5, H2A, H2B, H3, and H4. Histones H2A, H2B, H3, and H4 are known as the core histones: two copies of each of the four core histones assemble and are wrapped with ~146 bp of DNA to form the fundamental unit of chromatin known as the nucleosome. Histones were originally thought to solely function as a static scaffold for DNA packaging. Nowadays, it is evident that histones are highly dynamic proteins, undergoing multiple types of PTMs that regulate vital processes within the cell such as transcription, replication, recombination, and DNA repair. PTMs on histones mainly occur at the N-terminal tails and include (but are not limited to) acetylation, methylation, ubiquitination, poly(ADP-ribosyl)ation, and citrullination. These PTMs are described below, with a focus on modifications associated with cell death, which have been specifically related to autoimmune situations such as SLE (**Figure 1**).

### ACETYLATION

Acetylation and deacetylation of lysine residues at the N-terminal tails of histones play an important role in the regulation of transcription. Acetylation removes positive charges, thereby reducing the affinity between histones and DNA and maintaining an open and accessible conformation of DNA that is available for the binding of factors of the transcriptional machinery. Acetylation and deacetylation reactions are catalyzed by enzymes with respectively histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. Various studies have investigated the significance of histone acetylation in SLE. Hypoacetylation of histones H3 and H4 has been found in CD4+ T cells from SLE patients and splenocytes from MRL/lpr mice (67, 68). Treatment of these MRL/lpr mice with HDAC inhibitor trichostatin A reset the hypoacetylation of histones H3 and H4 and lead to an improved disease phenotype. In addition, it has been shown that mice deficient in HAT p300 develop an autoimmune disease similar to SLE in its pathological manifestations (69). Altered histone acetylation in unstimulated disease-relevant cells has been primarily linked to an altered gene expression. In contrast to unstimulated cells, apoptotic blebs or NETs from SLE patients contain hyperacetylated histones when compared to healthy donors (submitted data of our group). Hyperacetylation of histones appears to occur early during cell death. In the past, our group showed that hyperacetylated nucleosomes and acetylated histone peptides display an enhanced capability in activating the immune system (70). Hyperacetylated nucleosomes were able to mature bone marrow-derived DCs *in vitro*, which produced increased levels of IL-6 and TNF-α compared with DCs cultured in the presence of normally acetylated nucleosomes. In addition, DCs cultured in the presence of hyperacetylated nucleosomes were able to activate syngeneic T cells. Furthermore, subcutaneous administration of a specific triacetylated H4 peptide to pre-diseased MRL/lpr mice significantly enhanced mortality, proteinuria, skin lesions, and glomerular IgG depositions. In addition to a direct immunogenic affect, it is speculated that hyperacetylated histones in apoptotic blebs or NETs enhance the recruitment and binding of "dangerous" antimicrobial peptides such as LL-37, HMGB1, and HNPs. It has been shown that these antimicrobial peptides display a high immunogenic potential in NETs (61). Our group showed that autoantibodies in SLE patients frequently target acetylated epitopes in the N-terminal

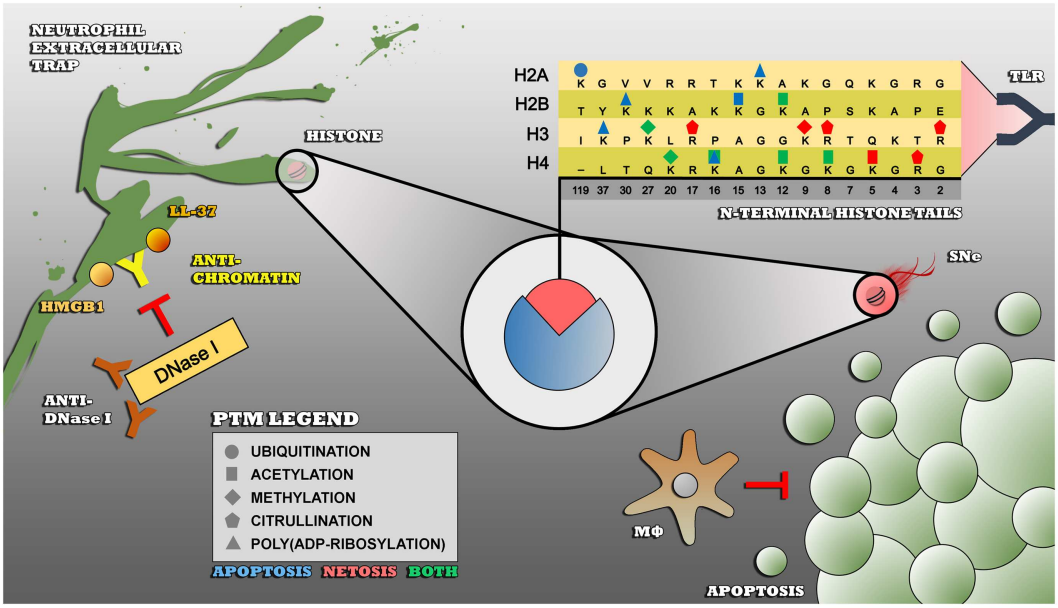


**FIGURE 1 | Impaired clearance of apoptotic cells and/or NETs leads to an enduring exposure of modified histones to the immune system – insufficiently cleared apoptotic cells by macrophages undergo secondary necrosis (SNe), thereby externalizing modified autoantigens such as histones that become recognized as foreign and dangerous by receptors of the innate immune system such as toll-like receptors (TLR)**. Modified histones are also highly present in NETs, that are also not properly cleared in SLE due to polymorphisms in the DNase I gene (not shown), inhibitory anti-DNase I autoantibodies, or NET-bound proteins such as HMGB1, LL-37, C1q (not shown), and anti-chromatin autoantibodies that prevent the accessibility for DNase I to the NET. The PTMs that are shown are associated with apoptosis (blue), NETosis, or both (green) and are linked to the autoimmune response in SLE.

tails of histones H2A, H2B, and H4. We characterized two monoclonal autoantibodies derived from a MRL/lpr mouse, KM-2 and LG11-2, that recognize tri-acetylated H4 at lysines 8, 12, and 16 and acetylated H2B (K12), respectively (70, 71). Both autoantibodies showed an increased reactivity with histones isolated from apoptotic cells and also from NETs (submitted data of our group), suggesting that these modifications are associated with apoptosis and NETosis. Another study also showed that IgG autoantibodies from histone-reactive SLE patients show high reactivity for acetylated H2B, whereas an increase in H4 acetylated at lysine 5 in NETs was demonstrated as well (36).

## METHYLATION

Histone methylation is the process by which methyl groups are transferred by histone methyltransferases (HMTs) to lysine or arginine residues of histones. Similar to acetylation, histone methylation regulates transcription and silencing of genes, depending on the target sites. Di-methylation of H3 at lysine 9 and mono-, di-, and tri-methylation of H4 at lysine 20 increases upon NETosis (36). Methylation of histone H4 at lysine 20 has also been associated with apoptosis (72). Our group recently demonstrated that autoantibodies present in the plasma from SLE mice and patients preferentially recognize tri-methylated H3 at lysine 27 (73). This latter reactivity was specific for SLE as there was hardly any reactivity in plasma samples from patients with rheumatoid arthritis (RA) or systemic sclerosis and healthy controls. Tri-methylation of H3 at lysine 27 also increases upon NETosis, as demonstrated by Liu et al. in ATRA-differentiated HL-60 cells (36). In our hands, this epitope is also highly present in NETs formed by primary neutrophils (submitted data of our group). Methylation of histones seems to be associated with an increased immunogenic potential of chromatin, similar to the aforementioned acetylation of histones, but additional research is required to unravel the exact mechanisms.

## UBIQUITINATION

The process of ubiquitination involves the conjugation of ubiquitin to other cellular proteins, thereby regulating a broad range of eukaryotic cell functions such as apoptosis, antigen processing, DNA transcription and repair, cell division, and immune responses. Ubiquitination may signal proteins for their degradation via the proteasome, alter their cellular location, affect their activity, and promote or prevent protein–protein interactions. In human, 10% of all H2A proteins is monoubiquitinated at lysine 119 (UH2A) (74). Autoantibodies against UH2A are frequently found in SLE and appear to be disease-specific (75). Between 60 and 70% of SLE patients are positive for anti-UH2A autoantibodies, compared to 10% of patients with systemic sclerosis. In RA, juvenile chronic arthritis, or Sjögren's syndrome, these autoantibodies are virtually absent. Deposits of UH2A have been identified in more than 50% of the renal biopsies from SLE patients with glomerulonephritis (76). Disappearance of UH2A (deubiquitination) is linked to late apoptotic processes and is likely to be disturbed in SLE (77, 78). Polymorphisms in the TNFAIP3 gene,

the gene that encodes for the deubiquitinating enzyme A20, are highly associated with SLE (79). These polymorphisms lead to a reduced expression of A20 and result in increased ubiquitination, as demonstrated by Jury et al. in T cells from SLE patients (80). Due to the inhibitory effect of A20 on the NFκB signaling pathway, TNFAIP3 polymorphisms also cause hyperactive NFκB signaling. The ribonucleoprotein SSA/Ro is, in addition to UH2A, also a ubiquitinated protein that is frequently target of SLE autoantibodies (81). Hyperubiquitinated histones released from late apoptotic cells or NETs are likely to display an increased antigenicity and immunogenicity, but the underlying mechanisms are not fully elucidated yet.

#### POLY(ADP-RIBOSYL)ATION

Poly(ADP-ribosyl)ation of proteins involves the addition of poly(ADP-ribose) moieties (PARs), mediated by poly(ADP-ribose) polymerases (PARPs). These reactions are involved in cell signaling and the control of many cell processes, including DNA repair, telomere maintenance, and apoptosis. Autoantibodies that bind to PARs or to the two zinc finger motifs of PARPs are frequently found in patients with autoimmune rheumatic and bowel diseases, and SLE (82–84). Anti-PARP autoantibodies do not significantly affect the enzyme activity of PAPRs, but prevent the cleavage of PARPs by caspase-3 (85). This cleavage is important in the proper execution of apoptosis. Inefficient cleavage of PARPs has shown to prolong cell survival *ex vivo* and may therefore cause failure to eliminate autoreactive lymphocytes and sustain autoimmune stimulation. Anti-PARP autoantibodies can penetrate cells in relative late stages of apoptosis, thereby neutralizing PARP activity. As a result of energy depletion, prolongation of cell survival may ultimately result in necrosis, thereby releasing poly(ADP-ribosyl)ated proteins that may possess high antigenic and immunogenic potential. It has been shown that oligo(ADP-ribosyl)ated histones are involved in the production of anti-PARP autoantibodies in SLE patients (86). Lysine 13 of H2A, lysine 30 of H2B, lysine 37 of H3, and lysine 16 of H4 have all been identified as ADP-ribose acceptor sites (87).

#### CITRULLINATION

Citrullination (also known as deamination) involves the conversion of arginine residues into citrulline, a process catalyzed by enzymes known as peptidylarginine deiminases (PADs). While most of the previously discussed PTMs are reversible and associated with reversible events involved in signal transduction, citrullination is not reversible. Citrullination has an important role in the normal function of the immune system, skin keratinization, the insulation of neurons, and the plasticity of the central nervous system including its essential role in gene regulation. In RA, autoantibodies to citrullinated proteins (anti-CCP) is considered a key pathogenic event. The presence of anti-CCP autoantibodies is a powerful biomarker that allows the diagnosis of RA to be made at a very early stage (88). Many groups investigated the role of NETs in the production of anti-CCP autoantibodies in RA- and PAD4-mediated citrullination of histones, which appear as the essential initiator for NETosis via decondensation of the chromatin (89). Khandpur et al. correlated accelerated NETosis in RA with anti-CCP levels (25), but this correlation has raised the following question: if NETosis, initiated by citrullination of histones, plays a pathogenic role in both RA and SLE, then why is the presence of anti-CCP autoantibodies highly sensitive and specific for RA only and not for SLE? Hence, anti-CCP autoantibodies are present in only 10–30% of SLE patients compared to 80–90% of RA patients (90, 91). Interestingly, SLE patients with arthritis are significantly more positive for anti-CCP than those without arthritis, suggesting that these autoantibodies have a predictive value for the development of arthritis in SLE (92). A groundbreaking publication by Romero et al. questions the contribution of NETs to anti-CCP production (93). This group showed that proteins highly present in RA synovial fluid cells become hypercitrullinated due to membranolysis of these cells by perforins and the membrane attack complex (MAC) of the complement system. This membranolysis results in a massive calcium influx that activates PADs to citrullinate various substrates, such as vimentin, fibronectin, and α-enolase. Although it seems that citrullinated histones, present in NETs and to a lesser extent also in apoptotic blebs, do not exert an important role in the induction of anti-CCP autoantibody production, they may still play a pathogenic role. To our knowledge, studies about the immunogenic effect of citrullinated histones in NETs or apoptotic cells have not yet been conducted. The antimicrobial peptide LL-37 is highly present in NETs from SLE patients and has recently been found to be also a substrate of PADs (94). Citrullinated LL-37 showed to be more chemotactic to PBMCs and more pro-inflammatory compared to unmodified LL-37. Comparable results may also hold for citrullinated histones, although additional research is required.

## CONCLUDING REMARKS

The exact etiopathogenesis of SLE is far from understood. Many different environmental factors are believed to act together to induce SLE in those who are genetically predisposed. There is a growing body of evidence that shows that disturbances in two cell death pathways, apoptosis and NETosis, are causative for initiating the disease and amplifying existing disease. However, the relative contribution of apoptosis and NETosis to the genesis of SLE is unclear and is likely to differ from patient to patient. Regardless of this, it is assumed that disturbances in both cell death pathways interact with each other and create multiple positive feedback loops that lead to chronification or exacerbation of the disease (**Figure 2**). Despite the heterogeneity in the underlying (molecular) defects and pathways that cause SLE, it appears that accumulation of apoptotic material and NETs in tissues is the common denominator between all patients with SLE. Enrichment of protein modifications, and in particular specific histone modifications, in not efficiently cleared apoptotic cells or NETs may generate neoantigens/danger signals with an increased antigenic and immunogenic potential. In addition to PTMs, another process to be considered in the generation of neoantigens/danger signals is proteolytic cleavage of histones and other chromatin-associated proteins by for example caspases, neutrophil elastase, and/or cathepsins. It is conceivable that chromatin-derived PTMs and/or cleavage products, related to apoptosis and/or NETosis, specifically ligate to receptors on antigen-presenting cells, thereby activating these cells and resulting in their immunogenic presentation. Improving or intensifying the clearance of apoptotic cells

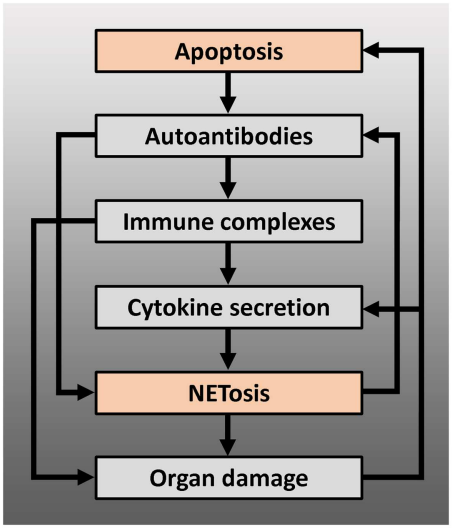


**FIGURE 2 | Positive feedback loops arising from the interaction between apoptosis and NETosis, leading to chronification and/or exacerbation of the disease – modified autoantigens, derived from apoptotic cells, may be presented by antigen-presenting cells to autoreactive T cells, which can lead to production of autoantibodies by B cells, including anti-dsDNA or anti-RNP antibodies**. These autoantibodies can induce NETosis or form immune complexes with their antigen. Immune complexes deposit on basal membranes, and incite a local inflammation (organ damage), or stimulate plasmacytoid dendritic cells to produce IFN-α and other pro-inflammatory cytokines. Pro-inflammatory cytokines such as IL-1β, TNF-α, or IFN-α induce NETosis or prime neutrophils for NETosis: NETs may serve as B cell autoantigens and lead to further autoantibody production or directly cause organ damage. Proteins from neutrophil granules, present in NETs, have shown to be highly toxic to glomerular structures and endothelium. Endothelial or glomerular damage causes further production of pro-inflammatory cytokines and leads to a new load of apoptotic cells.

and/or NETs may prevent the formation of immunogenic nuclear autoantigens. In addition, neutralizing and tolerizing strategies using specific chromatin-derived PTMs and/or cleavage products related to apoptosis and/or NETosis may represent future therapies for SLE.

## ACKNOWLEDGMENTS

This study was supported by grants from the Dutch Arthritis Association (grant 09-1-308; Johan van der Vlag), Dutch Kidney Foundation (KSBS 12.073; Elmar Pieterse/Johan van der Vlag), RadboudUMC Honours Academy (Elmar Pieterse).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 26 February 2014; accepted: 28 March 2014; published online: 09 April 2014. Citation: Pieterse E and van der Vlag J (2014) Breaking immunological tolerance in systemic lupus erythematosus. Front. Immunol. **5**:164. doi: 10.3389/fimmu.2014.00164 This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 01 July 2014
doi: 10.3389/fimmu.2014.00302

# Eosinophils in the lung – modulating apoptosis and efferocytosis in airway inflammation

***Jennifer M. Felton*, Christopher D. Lucas, Adriano G. Rossi and Ian Dransfield***

*MRC Centre for Inflammation Research, Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, UK*

***Edited by:***
*Luis Enrique Munoz, Friedrich-Alexander University, Germany*

***Reviewed by:***
*Masaaki Murakami, Hokkaido University, Japan*
*Fulvio D'Acquisto, Queen Mary University of London, UK*

****Correspondence:***
*Jennifer M. Felton, MRC Centre for Inflammation Research, Queen's Medical Research Institute, The University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK*
*e-mail: j.m.felton@sms.ed.ac.uk*

Due to the key role of the lung in efficient transfer of oxygen in exchange for carbon dioxide, a controlled inflammatory response is essential for restoration of tissue homeostasis following airway exposure to bacterial pathogens or environmental toxins. Unregulated or prolonged inflammatory responses in the lungs can lead to tissue damage, disrupting normal tissue architecture, and consequently compromising efficient gaseous exchange. Failure to resolve inflammation underlies the development and/or progression of a number of inflammatory lung diseases including asthma. Eosinophils, granulocytic cells of the innate immune system, are primarily involved in defense against parasitic infections. However, the propagation of the allergic inflammatory response in chronic asthma is thought to involve excessive recruitment and impaired apoptosis of eosinophils together with defective phagocytic clearance of apoptotic cells (efferocytosis). In terms of therapeutic approaches for the treatment of asthma, the widespread use of glucocorticoids is associated with a number of adverse health consequences after long-term use, while some patients suffer from steroid-resistant disease. A new approach for therapeutic intervention would be to promote the resolution of inflammation via modulation of eosinophil apoptosis and the phagocytic clearance of apoptotic cells. This review focuses on the mechanisms underpinning eosinophil-mediated lung damage, currently available treatments and therapeutic targets that might in future be harnessed to facilitate inflammation resolution by the manipulation of cell survival and clearance pathways.

**Keywords: eosinophil, lung, inflammation, apoptosis, phagocytosis, allergy, airway, resolution**

## INTRODUCTION

In response to tissue injury or the presence of micro-organisms, initiation of host protective mechanisms associated with the acute inflammatory response can also cause damage to the surrounding tissue. The release of proteases, glycosidases, and reactive oxygen/nitrogen species can be particularly destructive in the lung, where disruption of the normal tissue architecture compromises efficient gaseous exchange. A corollary of this close relationship between inflammation and tissue injury is that successful resolution of inflammation is crucial to optimal restoration and maintenance of lung function.

The detection of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) via their cognate receptors leads to the production of pro-inflammatory mediators including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, and IL-6. Well characterized chemoattractants such as complement fragments (e.g., C3a and C5a), lipids [e.g., leukotriene $B_4$ ($LTB_4$) and platelet-activating factor (PAF)], and chemokines [e.g., IL-8 (CXCL8), MCP-1 (CCL2), and eotaxin (CCL11)] act to recruit and/or activate inflammatory cells. Together these mediators rapidly perpetuate inflammation via the activation of vascular endothelial cells, increased vascular permeability, and edema, concurrent with the recruitment of granulocytes at the site of injury. In this review, we will discuss the mechanisms controlling acute lung inflammation, pathological conditions where the regulation of inflammation has gone awry and discuss the current and future treatments that could promote the successful resolution of inflammation.

## POLYMORPHONUCLEAR GRANULOCYTES: CRITICAL EFFECTORS OF THE INNATE IMMUNE RESPONSE

Neutrophils and eosinophils are key immune cells in the host defense against invading bacteria and parasites. Excessive recruitment, uncontrolled activation, and defective removal of these cells play a prominent role in the initiation and propagation of a number of chronic inflammatory conditions (1). Apoptosis, a major form of programed cell death, is a fundamental process regulating the tissue longevity of inflammatory cells. Apoptosis provides an efficient non-inflammatory mechanism for the removal of potentially damaging cells and cellular content from the inflamed site by resident or recruited monocyte/macrophage populations (2) or by "non-professional" phagocytes such as epithelial cells (3). The observation of failed apoptotic cell clearance seen in a number of chronic inflammatory diseases, including asthma, bronchiectasis, and chronic obstructive pulmonary disease (COPD) provides strong evidence that granulocyte apoptosis and non-inflammatory clearance has a key role in the resolution of inflammation.

Neutrophils are continuously generated from pluripotent stem cells in the bone marrow and are released into the circulation in large numbers [up to $2 \times 10^{11}$ cells/day (4)]. Once appropriately triggered, circulating neutrophils or those mobilized from the large marginated pools in the lungs, liver, spleen, and bone

marrow (5, 6), can be rapidly recruited to the inflammatory site and engage a number of effector mechanisms to destroy invading pathogenic organisms. This distinctive machinery includes a combination of reactive oxygen species (ROS) generation, the release of a cocktail of cytotoxic and proteolytic molecules, phagocytosis, and NETosis (the formation of extracellular chromatin traps) to destroy invading pathogenic organisms. Recruited neutrophils can undergo apoptosis which is associated with the "disabling" of secretion of their potentially harmful granule content thereby preventing damage to the surrounding tissues (7). The removal of these apoptotic cells by recruited macrophages or other local phagocytes, including airway epithelial cells (3), is believed to facilitate the resolution of inflammation. In addition to apoptotic cell death, a number of other forms of neutrophil cell death have been documented, including necrosis, NETosis, autophagocytic cell death, necroptosis, oncosis, and pyroptosis [reviewed in Ref. (8, 9)]. Although the impact of these alternative forms of cell death on the resolution of inflammatory responses is less clear, several are believed to be predominantly pro-inflammatory. As well as local cell death recent studies have provided evidence that recruited granulocytes can also undergo reverse migration away from the site of inflammation (10–12), although the consequences of this on inflammatory processes requires further investigation.

Eosinophils are also derived from granulocytic precursor populations in the bone marrow and are readily recruited from residence within hematopoietic and lymphatic organs such as the lymph nodes, thymus, spleen, and bone marrow (13) via the vasculature to the site of injury in response to parasitic or allergic inflammation (14). Historically, these cells were considered to play little role in immunoregulation, however, several lines of investigation have now shown eosinophils to be multifunctional granulocytes involved in the initiation and propagation of numerous inflammatory responses, including modulation of the adaptive immune response (14). Once at the site of injury, eosinophil degranulation contributes to both the removal of the inflammatory stimuli and also the propagation of inflammation. Eosinophil-derived granules contain a wide range of proteins including, major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin, which are known to be cytotoxic to airway epithelial cells (15, 16). Eosinophils have also been shown to undergo "traditional" extracellular trap formation (ETosis) termed EETosis (17) as well as facilitating the extracellular release of mitochondrial chromatin in a ROS-dependent manner (13). Released mitochondrial DNA and eosinophil-derived granule proteins combine to form structures, which are capable of both binding and killing invading organisms *in vitro* and *in vivo* (13), indicating that eosinophils may play a previously unrecognized role in antimicrobial defense. The fate of tissue eosinophils includes apoptosis (18) and subsequent clearance by phagocytes, although alternative fates have also been reported.

### APOPTOTIC PATHWAYS

There are two major pathways of apoptosis. The *intrinsic* pathway is characterized by a conformational change in pro-apoptotic Bcl-2 protein family members, resulting in outer mitochondrial membrane pore formation. The subsequent release of cytochrome *c* leads to formation of a complex with apoptotic protease-activating factor-1 (APAF-1), which then activates the downstream caspases that facilitate apoptosis. In contrast, the *extrinsic* pathway is triggered by cell surface death receptor trimerization resulting in the activation of Fas-associated protein with death domain (FADD) and TNF-receptor type 1-associated death domain protein (TRADD), which is responsible for the autocatalytic activation of initiator and effector caspases leading to the synchronized molecular alterations and morphological changes associated with apoptosis. Thus, the result of these two divergent pathways is the activation of intracellular caspases (a family of cysteine–aspartic proteases), which represents a hallmark event in apoptosis [reviewed in Ref. (8, 19, 20)].

## AIRWAY INFLAMMATION

### NORMAL LUNG STRUCTURE

The lung is made of up three distinctly different anatomical areas, the proximal cartilaginous airways, distal bronchioles, and alveoli (21). The trachea and main bronchi form the proximal cartilaginous airways and are responsible for the conduction of inhaled air, during which the proximal pseudostratified epithelium provides defense against invading pathogens and environmental toxins. In contrast, the epithelium of the distal airways becomes more columnar and is populated by a large number of ciliated epithelial cells and mucus-secreting goblet cells (22) – aiding the entrapment and further removal of unwanted inhaled particles (23). Two types of cells make up the alveolar epithelium; type 1 cells, which facilitate gaseous exchange, and the type 2 cells produce numerous secretory vesicles filled with surfactant material, including surfactant-associated protein C (24). Thus, in a normal lung the architectural structure of the tissue works to provide the most efficient environment for gaseous exchange.

Due to the large surface area and constant barrage of pathogens and debris found in the air, the lungs have developed efficient mechanisms for the recognition of microbe-specific motifs. The respiratory tract is also unique in that it has both an external epithelial layer (the respiratory epithelium) and an internal endothelial layer in close apposition. Therefore, this unique structure could provide difficulties when attempting to pharmacologically target the tissue resident eosinophils rather than the airway-resident cells.

### NEUTROPHIL-DOMINANT AIRWAY INFLAMMATION

In tissue localized infection, the exposure of neutrophils to bacterial products or endogenous mediators leads to "priming" of function and facilitates chemotaxis toward the site of infection or injury. Up regulation of surface adhesion molecules (P-selectin, ICAM1, and VCAM1) on the vascular endothelial cells that interact with adhesion molecules on the neutrophil is required for the tethering, rolling, intravascular crawling, and transmigration of activated neutrophils from the circulation into the tissue to carry out their effector functions [reviewed in Ref. (4)]. Development and progression of two neutrophil-driven airway diseases; COPD, characterized by impaired airflow to the lungs as a result of an abnormal inflammatory response (25), and bronchiectasis, a chronic debilitating respiratory disease, characterized by a "vicious cycle" of permanently dilated airways, increased mucus production, and recurrent infections (26), have been linked to failed

resolution of inflammation (27–29). However, despite persistent neutrophil-driven inflammation, reduced bacterial clearance is also seen (30). Thus, failure to clear bacterial pathogens from the airways leads to a prolonged inflammatory response characterizing the vicious cycle of inflammation and infection described, with both neutrophil and bacterial derived products contributing to damage of the surrounding epithelial cells.

Currently prescribed treatments for COPD and bronchiectasis include β2-adrenergic receptor agonists (e.g., salmeterol and formoterol), anticholinergic therapies (e.g., tiotropium bromide), high dose inhaled glucocorticoids, theophylline and treatments to improve mucociliary clearance, and sputum expectoration. These drugs work to reduce symptoms, improve lung function, and exercise capacity in an attempt to return to normal health status (26, 31–34). Furthermore, as well as traditional anti-inflammatory effects including inhibition of ROS release, decreased adhesion to the vascular endothelium and reduced release of pro-inflammatory cytokines from macrophages (35), salmeterol has also been shown to reduce adherence of bacteria to airway epithelial cells (36, 37), demonstrating that it may be effective at treating both the underlying infections and resultant inflammatory response.

## EOSINOPHIL DOMINANT AIRWAY INFLAMMATION

Eosinophil dominant allergic inflammation is characterized by three distinct phases (**Figure 1**). On initial exposure of the airway to an allergen, the sensitization stage, allergens are taken up by dendritic cells either within the airway lumen or in the submucosa after penetrating the epithelial barrier. The antigens are then presented to naïve T cells, which differentiate and activate local B cells to produce IgE. Secreted IgE then binds to Fcε receptors on the surface of submucosal tissue resident mast cells, thus priming the immune system. On second exposure to the allergen, surface bound IgE becomes cross-linked leading to the activation of the tissue resident mast cells. Inflammatory mediators are then released and initiate the propagation of inflammation characterized as the second phase of allergic inflammation, the early-phase reaction. Release of histamine, $LTB_4$, TNF-α, IL-8, IL-13, CCL2, and VEGFA from mast cells leads to increased vascular endothelial permeability, promoting the recruitment and transmigration of granulocytes from the circulation into the tissue. IL-13, histamine and TNF-α also act directly on the goblet cells found within the airway epithelium, causing increased mucus production.

Transendothelial migration of eosinophils to the inflamed site marks the progression into the third stage of the inflammatory response – the late-phase reaction. This usually develops 6–9 h after allergen exposure. Continued secretion of eosinophil recruiting cytokines (e.g., GM-CSF, IL-5, and IL-3) by mast cells leads to the prolonged eosinophil infiltration, representing a major contributory factor to the initiation and maintenance of eosinophilic airway inflammation in asthma (19, 38) – key cytokines involved in eosinophil recruitment are summarized in **Table 1**. Subsequent eosinophil degranulation and release of intracellular cytotoxic contents such as eosinophil basic protein results in damage to airway epithelial cells with increased mucus production from goblet cells and airway bronchoconstriction as a result of IL-13 secretion lead to reduced airflow, airway damage, goblet cell hyperplasia, and disrupted tissue architecture and remodeling. Mast cell production of IL-8 and TNFα also triggers the recruitment of neutrophils and elastase release causing further tissue degradation.

**Table 1 | Key cytokines involved in eosinophil recruitment**.

| KEY CYTOKINES |
|---|
| Eotaxin-1 (CLL11) (47, 48) |
| GM-CSF (49) |
| Interleukin-5 (IL-5) (49) |
| Interleukin-3 (IL-3) (38, 49) |
| MCP-3 (48) |
| Eotaxin-2 (CCL24) (47) |
| RANTES (CCL5) (48–50) |
| MIP-1α (CCL3) (48, 50) |

Continuous exposure to allergens leads to persistent, chronic inflammation, which is associated with changes in tissue architecture and cell composition and extensive tissue remodeling. In particular, patients with chronic asthma develop increased goblet cell hyperplasia. Persistent residence of eosinophils and neutrophils within the submucosa results in the continuous production of pro-inflammatory cytokines, lack of inflammatory resolution, and a repetitive cycle of tissue injury and inflammation (**Figure 1**).

The most characteristic features of eosinophil dominant allergic asthma is bronchial inflammation leading to non-specific airway hyperreactivity (39), mucus plugging of airways, epithelial cells loss, mucus gland hyperplasia, epithelial basement membrane thickening, edema of the submucosa, smooth muscle hypertrophy, and inflammatory cell infiltration (40). *In vitro* studies have shown that eosinophil granule-derived proteins are partly responsible for the damage seen in asthma as eosinophil cationic protein and major basic protein are both cytotoxic to the bronchial epithelium (16) and have been shown to affect ciliary beat and function (41) and increase non-specific bronchial hyperreactivity (42), all of which are classical pathological findings of asthma (40). Interestingly, a number of studies using eosinophil-deficient mice (PHIL) have shown that eosinophils enhance airway mucus accumulation and hyperresponsiveness, collagen deposition, and smooth muscle hypertrophy (43, 44). However, a significantly increased mucus index was still observed in ovalbumin treated PHIL mice (43) suggesting that although eosinophils contribute substantially to airway remodeling, they are not obligatory for allergen-induced injury (44), indicating activation of both eosinophil-dependent and -independent mechanisms of injury after airway allergen challenge (43).

Current treatments for asthma include inhalable bronchodilators (β2-adrenergic agonists and anticholinergic drugs), leukotriene receptor antagonists, glucocorticoids, and theophylline (45, 46). The possible mechanisms of actions for these drugs are shown in **Table 2**.

Mast cells are known to play an important role in the propagation and pathogenesis of allergen-induced inflammatory disease (51, 52), due to their involvement in the sensitization stage,

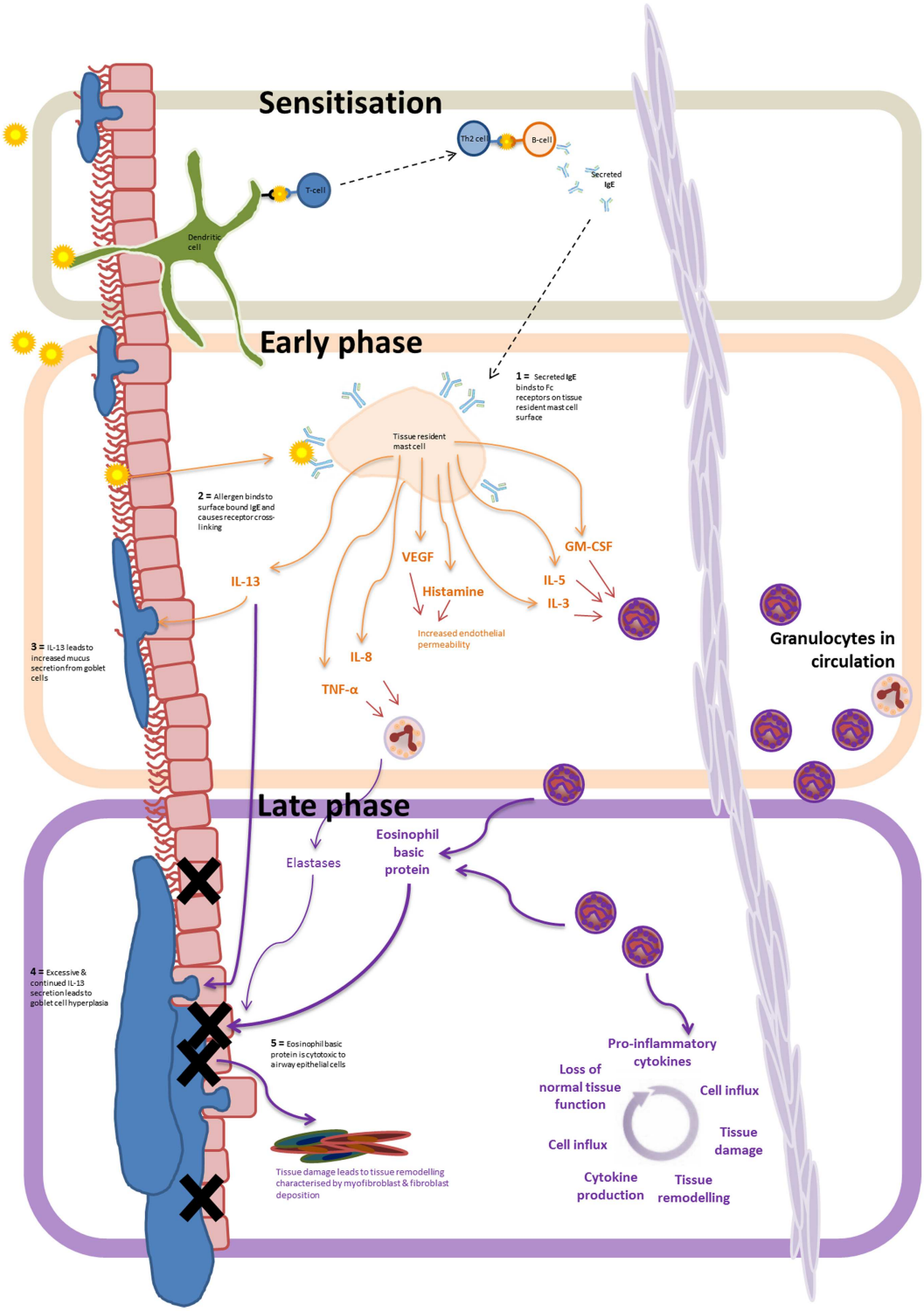


**FIGURE 1 | Allergen driven allergic inflammation: progression from the sensitization phase, to early and late inflammatory phase**. Airway exposure results in the allergen being taken up by submucosal dendritic cells. Antigen presentation to B cells, via T cell–dendritic cell interactions, leads to IgE release – marking the sensitization phase. IgE binding to Fcε receptors on the tissue resident mast cells leads to allergen-induced IgE receptor crosslinking and mast cell degranulation – marking the early-phase of the inflammatory response. The release of of $LTB_4$, TNF-α, IL-8, IL-13, CCL2, and VEGFA from mast cells results in eosinophil recruitment, increased vascular permeability, and increased mucus secretion by goblet cells. Continued exposure to the allergen, and infiltration of granulocytes to the inflamed tissue marks the progression to the late phase of the inflammatory response. Prolonged secretion of IL-13 and the release of intracellular cytotoxic granules by recruited eosinophils (and neutrophils) lead to continual tissue damage, mucus hypersecretion, and tissue remodeling resulting in the gradual loss of normal lung function.

**Table 2 | Mechanisms of action of agents currently used for routine treatment of allergic airways disease**.

| Mediator/drug | Biological response | Reference |
|---|---|---|
| Glucocorticoids | Alter pro- and anti-inflammatory cytokine balance | (55, 56) |
| | Enhances phagocytic capacity of macrophages and airway epithelial cells | (57–59) |
| | Promotes eosinophil apoptosis *in vitro* possibly via reduction of Mcl-1 levels | (1, 60) |
| β2-Adrenergic receptor agonists (e.g., salmeterol and formoterol) | Highly selective bronchodilator | (61–63) |
| | Reduce adherence of bacteria to airway epithelial cells | (36, 37) |
| Anticholinergic drugs (e.g., tiotropium bromide) | Effective bronchodilators | (32) |
| Leukotriene receptor antagonists | Prevent leukotriene induced bronchoconstriction, mucus hyper secretion, and airway inflammation | (64) |
| | Increase eosinophil apoptosis *in vitro* | (65) |
| Theophylline | Bronchodilator | (66, 67) |
| | Accelerates eosinophil apoptosis *in vitro* possibly by suppressing anti-apoptotic protein Bcl-2 levels | (68, 69) |
| | Reduced airway eosinophilia and ECP levels *in vivo* | (70) |

**Figure 1**. As these tissue resident cells are long lived and can survive repeated activation, the modulation of their proliferation and survival has been proposed as a potential therapeutic intervention for allergic disease (53). Recently, Wechsler et al. showed that thymol, a monocyclic phenolic plant compound with known antiseptic, antibiotic, antifungal, and antioxidant properties, was able to induce mast cell apoptosis *in vitro* and *in vivo* (54), thereby highlighting a potential pathway for modulating the allergic response through the manipulation of mast cell viability.

## FAILINGS IN CURRENT TREATMENTS: WHY NEW APPROACHES ARE NEEDED?

New approaches for the management of inflammatory airway diseases are urgently needed as current treatments are associated with a number of adverse health consequences after long-term use. For example, the management of allergic asthma is largely based around preventing exposure of the sensitized individuals to the allergen and treating with therapies which are directed toward alleviating and/or treating the symptoms of the disorder, such as inhaled glucocorticoids. However, this often poses a problem as a small subpopulation of asthma sufferers, often those with "neutrophilic" asthma, are noted to be steroid resistant (71), resulting in increased disease severity (72). Equally the undesirable side effects that arise from long-term use of glucocorticoids (the most common treatment prescribed for eosinophil dominant inflammatory conditions) include osteoporosis, hypertension, muscle atrophy, and delayed wound healing, all of which place limitations on use of glucocorticoid-based anti-inflammatory therapies.

### REGULATION OF GRANULOCYTE APOPTOSIS

As the resolution of inflammation likely depends on the apoptosis and phagocytosis of apoptotic granulocytes, research into the pharmacological manipulation of these processes is increasingly being recognized as an important area of research for the development of novel strategies to enhance the resolution of chronic inflammation (27, 73–76) and improve patient health.

The rates of granulocyte apoptosis are amenable to alteration by exogenous pharmacological compounds. Both the rates of neutrophil and eosinophil apoptosis can be accelerated by treatment with, soluble Fas ligand (Fas-L) (77), gliotoxin (78), and cyclin-dependent kinase inhibitors (CDKi) (73, 74, 79). Neutrophil apoptosis can also be delayed by pro-inflammatory cytokines (e.g., TNF-α and IL-1) (78), bacterial products [e.g., lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan] (80, 81), growth factors [e.g., granulocyte macrophage-colony stimulating factor (GM-CSF)] (75), and pharmacological agents including dibutyryl-cAMP (82, 83) and glucocorticoids (1, 84). TNF-α has been reported to have both pro- and anti-apoptotic effects on neutrophils, with early apoptosis and late survival seen during exposure of cultured cells. It is thought that at early time points (2–8 h) during *in vitro* culture a subpopulation of neutrophils undergo caspase-8 dependent apoptosis, with later survival (16–24 h) dependent upon an NF-κB mediated anti-apoptotic signaling pathway. Similarly, NF-κB inhibition in eosinophils allows TNF-α mediated apoptosis to predominate (85).

One important difference between the two granulocyte populations is that *in vitro* treatment of human granulocytes with glucocorticoids promotes eosinophil apoptosis, whereas it delays neutrophil apoptosis (5). It is also important to note that the survival effect of glucocorticoids on neutrophil longevity may be dependent on the environmental milieu (86, 87). Marwick et al. demonstrated *in vitro* that the pro-survival effects of glucocorticoids on neutrophils are dependent on oxygen levels, with severe hypoxia (1% oxygen) attenuating glucocorticoid-mediated neutrophil survival (87). This observation has important implications for the therapeutic efficacy of glucocorticoids when prescribed for neutrophil-dominant inflammatory conditions, due to the relatively high oxygen concentrations found in the lung.

One way that glucocorticoids are thought to mediate their anti-inflammatory effects is through the expression and function of the downstream effector molecule Annexin A1 (AnxA1) (86, 88). AnxA1 has been shown to promote human neutrophil apoptosis via dephosphorylation of the Bcl-2-antagonist of cell death (BAD) promoting cell death via the intrinsic pathway of apoptosis (89). *In vitro* investigations showed that endogenous AnxA1

was released by apoptotic neutrophils and glucocorticoid-treated macrophages, which then acts in both a para- and autocrine manner to promote the phagocytic clearance of apoptotic neutrophils (90, 91). Increased production of AnxA1 by innate immune cells following glucocorticoid administration reportedly leads to decreased neutrophil endothelial transmigration, increased neutrophil apoptosis and increased phagocytosis of apoptotic cells by macrophages (88, 92). This mechanism was further supported by *in vivo* experiments in which administration of an anti-AnxA1 antibody prevented glucocorticoid-induced resolution of inflammation, whereas treatment with AnxA1-derived peptides promoted the resolution of inflammation (86). Further work is required to define the role of AnxA1 in the resolution of eosinophilic inflammation.

A number of classically "pro-inflammatory" eosinophil recruiting cytokines, IL-25 (93), IL-33 (94), IL-3, IL-5, and thymic stromal lymphopoietin (TSLP) (95) have also been shown to delay the rate of eosinophil apoptosis (96). Due to their key role in the recruitment and activation and of eosinophils in inflammatory sites, there is interest in developing anti-cytokine therapies, such as anti-IL-5 antibodies [reviewed in Ref. (97)], as potential therapeutic targets. Historically, there has been a number of disappointing results surrounding anti-IL-5 treatments in humans, potentially as a result of the unique architecture of the lungs localizing their effect to the airway resident, rather than the tissue resident, eosinophils.

## NOVEL REGULATORS OF EOSINOPHIL APOPTOSIS

The regulation of cell apoptosis by pro- and anti-apoptotic Bcl-2 family members has been well documented (20). However, as granulocytes have a limited number of mitochondria (98), it was somewhat surprising when members of this protein family were found to modulate the regulation of granulocyte apoptosis. Eosinophils were found to express high levels of pro-apoptotic Bax molecules, and were also found to express a number of anti-apoptotic members of the Bcl-2 family (99). Mcl-1, an important anti-apoptotic protein in neutrophils (27), is also thought to play a predominant role in eosinophils, as previous work reported that Mcl-1 levels decreased in glucocorticoid-treated eosinophils, whereas they remained at a constant level in glucocorticoid-treated neutrophils (60), which may go some way to explain their differential effect on the two granulocyte populations.

Since granulocytes are considered to be terminally differentiated cells, the central role of active cyclin-dependent kinases (key regulators of the cell cycle) in the control of apoptosis was surprising. The structurally distinct CDKis R-roscovitine and AT7519 promoted apoptosis in a caspase-dependent manner (73, 76) by the down-regulation of intracellular Mcl-1 levels *in vivo* (79, 100, 101) and prevented GM-CSF-mediated up regulation of Mcl-1 (73). R-roscovitine was also shown to have pro-resolving effects *in vivo* in a number of models of inflammation (73) while AT7519 increased the percentage of apoptotic eosinophils as well as the percentage of macrophages containing apoptotic eosinophils in a mouse model of allergic pleurisy, indicating that AT7519 has the potential to resolve allergic inflammation by driving both eosinophil apoptosis and by increasing macrophage clearance of apoptotic cells (76). Evidence suggests that these CDKIs target CDK7 and CDK9, which are involved in transcription of key granulocyte survival proteins such as Mcl-1 (102). Further studies investigating the mechanisms underlying resolution of inflammation have also highlighted the importance of Mcl-1 in the regulation of granulocyte apoptosis. Flavones, polyphenolic plant-derived compounds, rapidly induced both eosinophil (103) and neutrophil apoptosis (101) *in vitro* even in the presence of powerful pro-survival mediators including LTA, GM-CSF (101), and IL-5 (103).

Another powerful driver of caspase induced eosinophil apoptosis is antibody crosslinking of sialic acid binding immunoglobulin-like lectin 8 (Siglec-8), a member of the Siglec immunoglobulin supergene family expressed only on the surface of human eosinophils, basophils, and mast cells (104). Siglec-8 cross-linking reduced eosinophil viability in a time- and concentration-dependent manner through the induction of caspase-mediated apoptosis. This was further confirmed by the use of pan (104) and selective caspase inhibitors (against caspase-8 and -9) (105), which completely inhibited Siglec-8 cross-linking induced apoptosis *in vitro*, while having no effect on spontaneous eosinophil apoptosis. Antibody crosslinking of the functional mouse ortholog, Siglec-F, was also shown to significantly reduce peripheral eosinophil number in a hypereosinophilic/chronic eosinophilic leukemic (HES/CEL) murine model, as well as induce eosinophil apoptosis *in vivo* (106). These data further highlight that regulation of eosinophil apoptosis using exogenous mediators could provide potential future therapeutic targets for eosinophilic disorders.

Pharmacological modulation of endogenous molecules involved in mediating the resolution of allergic inflammation is also a key area of research. Recently, Faustino et al. showed that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a major player in the resolution process, with *in vivo* administration of anti-TRAIL markedly reducing the number of apoptotic cells in the BAL fluid of chronic allergy induced mice (107). *In vivo*, treatment with recombinant TRAIL, in an established mouse model of allergic airway inflammation, also significantly augmented the number of apoptotic cells found in the BAL of OVA treated mice, compared to control (PBS treated mice), as well as decreasing the overall number of eosinophils found in the BAL (107), providing further evidence that the manipulation of eosinophil apoptosis may provide avenues for the discovery of novel therapeutics.

## LIPID MODULATION

Lipoxins, protectins, and resolvins are bioactive lipids synthesized from arachidonic, docosahexaenoic, and eicosapentaenoic acid, respectively (108, 109). They are key pro-resolution mediators, which act to selectively prevent granulocyte migration and increase the recruitment of phagocytic cells (110). Both lipoxin A4 and B4 were reported to inhibit neutrophil recruitment to an inflammatory site and lipoxin A4 was also shown to stimulate monocyte chemotaxis and promote macrophage uptake of apoptotic neutrophils *in vitro* (111, 112) and *in vivo* (113). Similar inhibitory effects of lipoxin A4 upon the migration and chemotaxis of eosinophils *in vivo* and the local generation of eotaxin and IL-5 have been reported (114, 115). Resolvins also have pro-resolution effects preventing transepithelial and

transendothelial migration of neutrophils *in vivo* (116), and stimulating the non-inflammatory phagocytosis of apoptotic neutrophils (117).

The significance of pro-resolving lipids in successful resolution of inflammation has also been noted in a number of non-allergic and allergic inflammatory conditions. Reduced levels of protectin D1 and lipoxin A4 are seen in the exhaled breath of patients after a severe and mild asthma exacerbation, respectively (118, 119). *In vivo*, mouse models have also provided insight into the role of pro-resolving lipids in the recruitment of granulocytes and augmentation of macrophage phagocytic capacity – resolvin E1 and lipoxin analogs were shown to reduce airway hyperresponsiveness (119), eosinophil number (118), and promote inflammation resolution in a mouse model of allergic asthma (119, 120). Given the pro-resolution roles of these lipid mediators, there is great interest in the development of them as therapeutics. However, as these endogenously produced molecules are traditionally locally active and rapidly inactivated the development of exogenously administered drugs with longer half-lives, which mimic endogenous compounds *in vivo* are needed to fill this pharmacological niche. Currently, the synthetic resolvin analog RX-10045, and naturally occurring small molecule lipid mediator RX-10001 and are under clinical examination for their use in a number of inflammatory diseases such as dry eye, asthma, retinal disease, and inflammatory bowel disease (109).

### MANIPULATION OF PHAGOCYTOSIS

One newly emerging approach to facilitate the resolution of inflammation is the pharmacological manipulation of the phagocytosis of apoptotic granulocytes (121). As well as their effects on granulocyte apoptosis, glucocorticoids are also known to augment macrophage phagocytic function (57), which may represent an approach to drive clearance of apoptotic cells from inflamed sites (122). Glucocorticoid-treated macrophages exhibit altered cytoskeletal regulation, with increased cell motility and expression of high levels of active Rac, a key protein involved in cell motility, mitosis, wound healing, and phagocytosis (123).

Glucocorticoids induce phagocytosis of apoptotic cells via Mer, a member of the Tyro-3/Axl/Mer (TAM) receptor tyrosine kinase family (58, 124, 125). TAMs are widely expressed vertebrate-specific receptor tyrosine kinases that confer the capacity for binding and subsequent phagocytosis of apoptotic cells, together with initiation of signals that regulate cellular function. TAM-deficient mice show defective clearance of apoptotic material by retinal pigment epithelial cells of the eye, Sertoli cells of the testis and also by myeloid cells. Interestingly, a number of autoimmune conditions are associated with impaired or failed clearance of apoptotic cells (126) and the absence of TAM receptors results in progressive loss of vision, reduced fertility, and development of overt autoimmunity (122, 127, 128). Recent studies demonstrate that Mer-mediated apoptotic cell clearance has a critical importance pathophysiologically in the lung, as inflammation in an LPS-induced lung injury model was amplified following Mer blockade (129), and conversely attenuated following up regulation of Mer-signaling by use of TAPI-0 (a specific inhibitor of Mer cleavage) (130). The expression of Mer on phagocytic populations present at the inflammatory site could also be induced by treatment with glucocorticoids (57) or liver X receptor agonists (131). Alternatively, blockade of cytokines that actively suppress Mer expression (e.g., interferon-gamma) could represent an alternative strategy for promoting Mer-dependent apoptotic cell clearance (132, 133). Recent evidence suggests that Mer is down regulated by inflammatory stimuli such as LPS or bleomycin via proteolytic cleavage from the phagocyte membrane (134). Specific inhibition of ADAM17 proteolytic activity (e.g., using KD-1X-73.5 or TAPI-0) prevents Mer down-regulation and is associated with increased clearance of apoptotic cells in both LPS and bleomycin models of lung injury (135), providing a potential therapeutic approach to increase Mer-dependent clearance mechanisms in inflammation. Definition of the molecular mechanisms of phagocyte–apoptotic cell interactions and regulation by glucocorticoids will provide opportunities to identify novel targets for therapeutic gain.

### EPITHELIAL CELL PHAGOCYTOSIS

In addition to the importance of therapies, which are able to modulate apoptotic cell phagocytic clearance, identification of the cell types which carry out this process in inflammatory airway conditions is of crucial importance. Induced death of airway epithelial cells as a result of exposure to environmental toxins, allergens, and pathogens has been observed and documented in patients with asthma (3). Thus, there is a need for a large population of local airway phagocytic cells to remove the apoptotic debris. There is mounting evidence that a number of "non-professional" phagocytes, including mammary epithelial (136) and microvascular endothelial cells (137) are also able to phagocytose apoptotic cells. Work published by Walsh et al. and Sexton et al. showed that bronchial epithelial cells are capable of recognizing and engulfing apoptotic eosinophils, suggesting a non-passive role of the airway epithelium in the resolution of eosinophilic inflammation in asthma (138, 139). More recently, Juncadella et al. showed that bronchial epithelial cells are also critically involved in the phagocytosis of apoptotic airway epithelial cells, which subsequently alters the production of anti-inflammatory cytokines and control of airway hyperresponsiveness in a murine model of allergic airway inflammation (3). Despite the potential for providing novel therapeutic approaches for the treatment of inflammatory diseases, little work has been done to investigate the potential for manipulation of the phagocytic ability of these cells in current models of inflammatory airway diseases.

## SUMMARY

In conclusion, recent research into the pharmacological manipulation of apoptosis and efferocytosis of apoptotic cells has provided novel insights into the treatment of inflammatory airway diseases, notably eosinophil dominant airway inflammation. This dual approach will open up new areas for therapeutic intervention, allowing the successful manipulation of inflammation resolution, as well as reducing the adverse effects associated with currently available treatments.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 01 May 2014; paper pending published: 19 May 2014; accepted: 15 June 2014; published online: 01 July 2014.*
*Citation: Felton JM, Lucas CD, Rossi AG and Dransfield I (2014) Eosinophils in the lung – modulating apoptosis and efferocytosis in airway inflammation. Front. Immunol. **5**:302. doi: 10.3389/fimmu.2014.00302*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 01 August 2014
doi: 10.3389/fimmu.2014.00354

# Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications

***Zsuzsa Szondy[1]*, Éva Garabuczi[1], Gergely Joós[1], Gregory J. Tsay[2] and Zsolt Sarang[3]***

[1] *Department of Dental Biochemistry, Faculty of Dentistry, University of Debrecen, Debrecen, Hungary*
[2] *Department of Internal Medicine, Faculty of Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan*
[3] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary*

***Edited by:***
*Martin Herrmann, Universitätsklinikum Erlangen, Germany*

***Reviewed by:***
*Amiram Ariel, University of Haifa, Israel*
*Paul G. Winyard, University of Exeter, UK*

****Correspondence:***
*Zsuzsa Szondy, Department of Biochemistry and Molecular Biology, University of Debrecen, Nagyerdei krt. 98, Debrecen H-4012, Hungary*
*e-mail: szondy@med.unideb.hu*

In healthy individuals, billions of cells die by apoptosis every day. Removal of the dead cells by phagocytosis (a process called efferocytosis) must be efficient to prevent secondary necrosis and the consequent release of pro-inflammatory cell contents that damages the tissue environment and provokes autoimmunity. In addition, detection and removal of apoptotic cells generally induces an anti-inflammatory response. As a consequence improper clearance of apoptotic cells, being the result of either genetic anomalies and/or a persistent disease state, contributes to the establishment and progression of a number of human chronic inflammatory diseases such as autoimmune and neurological disorders, inflammatory lung diseases, obesity, type 2 diabetes, or atherosclerosis. During the past decade, our knowledge about the mechanism of efferocytosis has significantly increased, providing therapeutic targets through which impaired phagocytosis of apoptotic cells and the consequent inflammation could be influenced in these diseases.

**Keywords: apoptotic cell, phagocytosis, inflammation, autoimmunity, atherosclerosis, obesity, type 2 diabetes, therapy**

## INTRODUCTION

Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional and by non-professional neighboring phagocytes, is a key mechanism in maintaining tissue homeostasis. Every day, billions of our cells die and get cleared without initiating inflammation and an immune response (1). Proper clearance of dead cells also contributes to the initiation of tissue repair processes following injury (2–4). In addition, efficient removal of apoptotic neutrophils is also a key event in the resolution of inflammation (5).

Increasing evidence suggest that improper clearance of apoptotic cells, being the result of either genetic anomalies and/or a persistent disease state, contributes to the establishment and progression of a number of human diseases via affects on the maintenance of tissue homeostasis, tissue repair, and inflammation (6). Autoimmune disorders, in which both animal models and human research indicate a strong relationship between improper clearance and the development of the disease, represent the best characterized example of such diseases. The regulated nature of apoptotic cell death normally prevents the leakage of the immunogenic intracellular contents. If, however, these cells are not promptly cleared, they undergo secondary necrosis leading to the release of the intracellular antigens and DNA, which in the long-term provoke an auto-inflammatory response (7). Thus, in most of the knock out mice in which efferocytosis is impaired, systemic lupus erythematosus (SLE) like autoimmunity develops (8–13). Human SLE is also accompanied by improper efferocytosis (7), and can develop also as a result of a genetic deficiency of the phagocytosis process (13).

While in SLE improper clearance of apoptotic cells affects all the tissues, in several chronic inflammatory respiratory diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, and asthma, increased numbers of apoptotic cells are seen only in the sputum and lung tissue (14). Though so far no evidence was provided for a definite linkage between genetic anomalies affecting efferocytosis and lung disease, inefficient apoptotic clearance in the lung was detected in all these respiratory diseases (15).

Macrophages play a key role in the development of atherosclerosis, and impaired clearance of apoptotic macrophages characterizes the late plaques, in which uncleared apoptotic cells undergo secondary necrosis leading to the formation of an unstable necrotic core and the maintenance of inflammation (16). Impaired efferocytosis, however, might also contribute to the development of the disease, as knock out mice deficient in efferocytosis are prone to develop atherosclerosis on LDL or ApoE null genetic backgrounds (17–20). An excess of apoptotic cells was detected in a numerous neurodegenerative diseases as well, such as Parkinson's, Alzheimer's, and Huntington's disease (21). Though the elevated levels of apoptotic cells might also be the result of an increased neuronal cell death, in these diseases loss of signaling by fractalkine (an apoptotic cell "find me" signal) resulted in an increase in the number of dying cells and worsening of the disease (22).

Interestingly, type 2 diabetes and obesity were also shown to be associated with impaired phagocytosis of apoptotic β-cells in the pancreas in autoimmune diabetes-prone rats (23) and in ob/ob and db/db mice (24). The phenomenon seems to be related to an enhanced saturated and/or decreased ω-3 fatty acid composition of the plasma membrane, which leads to a decreased

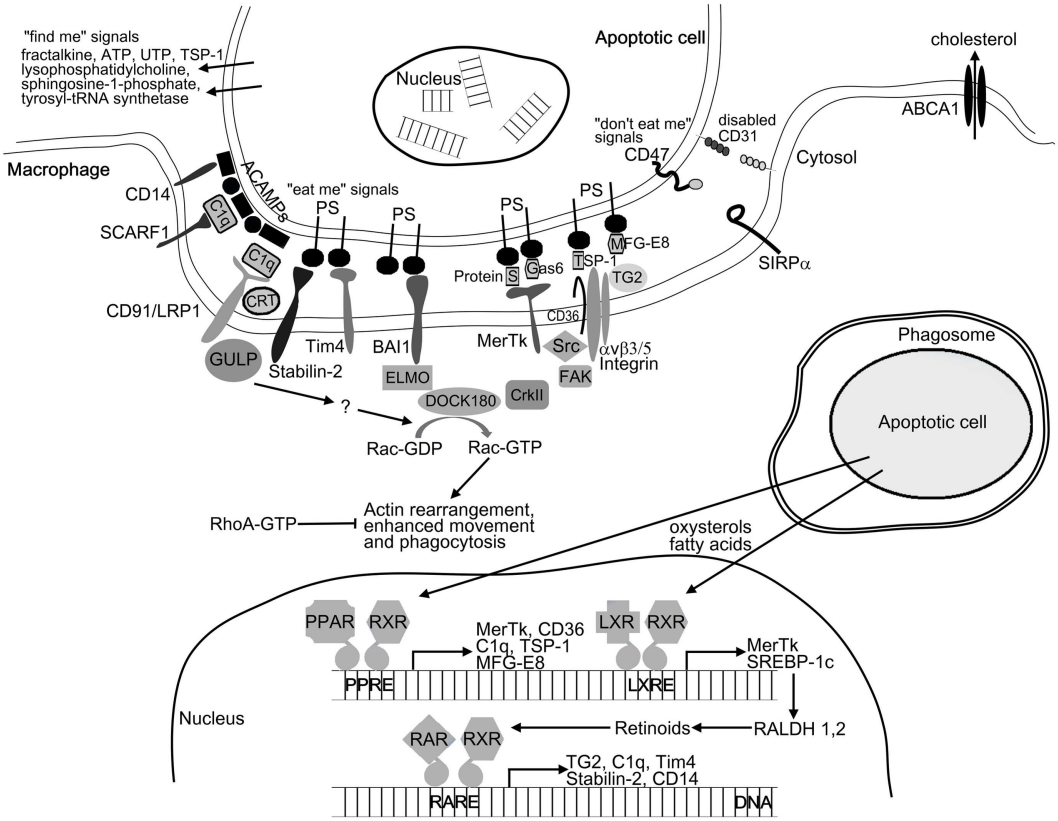


**FIGURE 1 | Mechanism of apoptotic cell clearance**. For initiating phagocytosis apoptotic cells release "find me" signals for the phagocytes. After finding the recognition of apoptotic cells by phagocyte receptors is mediated by the display of "eat me" signals (e.g., PS and ACAMPs) and the disappearance of the so-called "do not eat me" signals (e.g., CD31 and CD47) on the apoptotic cell surface. Among others, these receptors include the PS receptors (Tim4, stabilin-2, and BAI1) and receptors such as MerTk, SCARF1, CD36, and integrin αvβ5 together with TG2 recognizing apoptotic cells through bridging molecules (e.g., TSP-1, C1q, Gas6, MFG-E8, and protein S). Binding of apoptotic cells to the phagocytic receptors triggers two evolutionary conserved signaling pathways. MerTk, BAI1, and αvβ3/5 receptors will activate the DOCK180/CrkII/ELMO complex, while CD91/LPR1 and stabilin-2 will activate the adaptor protein GULP. Both pathways converge on the small GTPase Rac, which initiates actin rearrangement and phagocytosis. Following engulfment, apoptotic cell derived lipids (oxysterols and fatty acids) trigger the lipid-sensing LXR and PPAR receptors leading to enhanced retinoid production. Retinoid receptors together with LXR and PPARs upregulate a number of phagocytic receptors to further enhance the engulfing capacity of macrophages under conditions when the rate of apoptosis is increased.

phosphatidylinositol 3-kinase activation during the uptake of apoptotic cells (24).

## MECHANISMS CONTRIBUTING TO EFFICIENT PHAGOCYTOSIS OF APOPTOTIC CELLS

### "FIND ME" AND "EAT ME" SIGNALS

To ensure effective removal, apoptotic cells recruit phagocytes by releasing various soluble "find me" signals. These signals include lysophosphatidylcholine (25), CX3CL1/fractalkine (26), sphingosine-1-phosphate (27), the nucleotides ATP and UTP (28), thrombospondin-1 (TSP-1) (29), and cleaved human tyrosyl-tRNA synthetase (30). Upon arrival at the target cells, phagocytes must distinguish between apoptotic and viable cells. Apoptotic cells display apoptotic cell-associated molecular patterns (ACAMPs), which includes the appearance of "eat me" signals on their cell surface (5). These can bind either directly or through bridging molecules to receptors on phagocytes (**Figure 1**). Externalization of phosphatidylserine (PS) on the outer leaflet of the cell membrane is the best characterized "eat me" signal during apoptosis. The T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4), stabilin-2, and brain-specific angiogenesis inhibitor 1 (BAI1) were reported to directly recognize PS on dying cells (31–33), while other receptors such as Mer tyrosine kinase (MerTk), scavenger receptor SCARF1, CD36, and integrin αv/β3/β5 together with CD36 or tissue transglutaminase (TG2) recognize apoptotic cells through bridging molecules. Gas6 and protein S were found to facilitate apoptotic cell clearance by recognizing PS on apoptotic cells and MerTk receptor on phagocytes (34, 35). TSP-1 and milk-fat globulin-E8 (MFG-E8) also bind to PS and are recognized by the integrin αv/β3/CD36 or integrin αv/β3/TG2 receptor complexes, respectively (36–38). The collectin family member serum protein C1q also serves as a bridging molecule by recognizing annexin A2 and A5 on the apoptotic cells (39) and binding either SCARF1 scavenger receptor or the calreticulin associated LRP1/CD91 receptor on phagocytes (39, 40). The LPS coreceptor CD14 can also act as a tethering receptor for apoptotic

cells, albeit its exact ligand remains unknown (41). Distinguishing between apoptotic and viable cells is further ensured by the "do not eat me" signals, which inhibit the uptake of living cells. CD47, activating SIRPα receptor, is one of these signals being expressed on living cells but altered or diminished on apoptotic cell surface (42). Additionally, homophilic interaction between CD31 on the target cells and macrophages was shown to mediate cell detachment from phagocytes, thus inhibiting phagocytosis of living cells (43).

#### ENGULFMENT AND INGESTION OF THE APOPTOTIC CORPSES

Uptake of the apoptotic cells requires the reorganization of the actin filament network, which drives the movement of the cell, formation of the phagocytic cup and the phagosome. This process is regulated by the small GTPases RhoA, Cdc42, and Rac. While RhoA activation was found to inhibit apoptotic cell phagocytosis, Cdc42, and Rac were shown to enhance it (44). Phagocytic receptors activate two evolutionary conserved pathways both converging on the activation of Rac-1, a small GTPase (45) (**Figure 1**). The first pathway is initiated by MerTk or integrin αv/β5 receptors (46, 47), resulting in association of the adaptor protein ELMO with the Rac GEF DOCK180 forming a bipartite GEF (48). Recruitment of the ELMO/DOCK180 complex to the cell membrane might require the adaptor protein CrkII, but binding of ELMO to the carboxyl terminus of BAI1 also recruits DOCK180 to the phagocytic membranes (33). The second pathway activating the Rac is initiated by LRP1 (CD91) (49) or by stabilin-2 receptors followed by recruitment of the adaptor protein GULP (50). Further steps, resulting in the activation of Rac are still unclear. The newly formed phagosome must fuse with lysosomes to degrade the dead cells. Recently, several autophagic genes were described to participate in phagosome maturation (51, 52). Following phagolysosomal fusion, lysosomal enzymes degrade the content of phagolysosomes. Lysosomal cathepsin protease CPL-1 was found to be indispensable in the digestion of apoptotic cell derived proteins (53), while lysosomal DNase II degrades the DNA content (54).

#### REPROGRAMING OF PHAGOCYTES BY APOPTOTIC CELL CONTENT

Engulfment of apoptotic cells delivers excess materials to the phagocytes. Some of these materials can be completely degraded, while the excess of non-digestible cholesterol is removed via ATP-binding cassette (ABC) transporters (**Figure 1**). Both PS (55) and lipid-sensing nuclear receptors (56, 57) can upregulate the levels of the ABCA1 transporter. The ingested macromolecules provide the extra energy required for prolonged phagocytosis. However, if too much energy is generated, engulfing cells upregulate the mitochondrial uncoupling protein 2 (UCP2) and dissipate $H^+$ gradient to reduce mitochondrial membrane potential (58). UCP2 also decreases reactive oxygen species formation.

To ensure efficient long-term phagocytosis, apoptotic cells reprogram macrophages not only by altering their metabolism but also by increasing the expression of a number of phagocytic receptors via activating peroxisome proliferator-activated receptor (PPAR)δ/γ and liver X receptor (LXR)α/β receptors by their lipid content (59–61). This process is partially mediated via upregulation of endogenous retinoid synthesis (62, 63).

While the phagocytosis of a variety of pathogenic targets normally triggers a pro-inflammatory response in macrophages, ingestion of apoptotic cells by macrophages induces an anti-inflammatory phenotype. The earliest anti-inflammatory activity of the apoptotic cell is manifest as an immediate-early inhibition of macrophage pro-inflammatory cytokine gene transcription and is exerted directly upon binding to the macrophage (64). Subsequently, both nuclear receptors (65, 66) are activated and soluble mediators are released from macrophages, which act in a paracrine or autocrine fashion to amplify and sustain the anti-inflammatory response (67, 68). During the resolution of inflammation the reprogramed macrophages appear as pro-resolving CD11b$^{low}$ macrophages (69) that express immunoregulatory 12/15-lipoxygenase (70) involved in the formation of pro-resolving lipid mediators, termination of phagocytosis, and emigration to lymphoid organs (69) required for the proper termination of the inflammatory program. This process is regulated by the expression of a typical chemokine receptor D6 on the surface of apoptotic neutrophils (71).

Since improper efferocytosis might contribute to both the initiation and the maintenance of human diseases, enhancing phagocytosis might provide a therapeutic possibility to influence the progression of these diseases.

## THERAPEUTIC POSSIBILITIES FOR ENHANCING EFFEROCYTOSIS IN DISEASES IN WHICH CLEARANCE OF APOPTOTIC CELLS IS IMPAIRED

#### AFFECTING RECOGNITION AND BINDING OF APOPTOTIC CELLS

If lack of sufficient MFG-E8 production leading to improper efferocytosis participates in the pathomechanism of a disease, providing MFG-E8 in recombinant protein form to the site of acute inflammation might enhance the efficiency of efferocytosis. Indeed, a decreased MFG-E8 expression was found in inflamed colons during the acute phase of murine experimental colitis, and intrarectal treatment with recombinant MFG-E8 ameliorated colitis by reducing inflammation and improving disease parameters (72). Alternatively, both prolactin (73) and glucocorticoids (74) can enhance MFG-E8 production providing a theoretical possibility for enhancing its expression in macrophages systematically.

MFG-E8 contains a PS binding domain, as well as an arginine–glycine–aspartic acid (RGD) motif, which enables its binding to integrins. Opsonization of the apoptotic cells and binding to integrins on the surface of phagocytic cells, mediates the engulfment of the dead cell. Based on this observation, an RGD–anxA5 was designed, and it was shown that introduction of RGD transformed the annexin A5, a molecule that binds to PS of apoptotic cells, from an inhibitor into a stimulator of efferocytosis (75). While recombinant MFG-E8 or the RGD–anxA5 could be utilized in acute inflammation, long-term administration of MFG-E8 leads to obesity, because it stimulates the fatty acid uptake of adipocytes (76). It is an open question, whether chronic administration of RGD–anxA5 would have the same side effects.

While MFG-E8 acts as a bridging molecule for integrins, Gas6, and protein S are bridging molecules for MerTk. Thus in cases, where MerTk plays a driving role in efferocytosis, such as cardiac repair after myocardial infarction (4), provision of Gas6 or protein S could similarly accelerate phagocytosis of apoptotic

cells and tissue repair. Glucocorticoids enhance phagocytosis by making efferocytosis MerTK dependent (77), thus combining glucocorticoids and Gas6 or protein S might have a synergistic effect.

Other bridging molecules, such as collectins, were also reported to promote efferocytosis. Macrolide antibiotics, which have wide-ranging anti-inflammatory effects, were found to enhance efferocytosis by enhancing the expression of collectins (78). The therapeutic potential of these drugs has already been recognized, as they are successfully used in the treatment of COPD, cystic fibrosis, or asthma (79).

### TARGETING LIPID-SENSING NUCLEAR RECEPTORS WITH THE AIM OF INCREASING THE EXPRESSION OF PHAGOCYTIC RECEPTORS OR THEIR BRIDGING MOLECULES

Since nuclear receptor signaling is strongly associated with enhanced efferocytosis and suppression of inflammation, glucocorticoids, PPARγ, PPARδ, and LXR agonists or retinoids are logical therapeutic targets in diseases in which efferocytosis is impaired.

Glucocorticoids, the most widely used anti-inflammatory drugs, were shown to enhance phagocytosis of apoptotic cells by increasing the expression of the phospholipid binding protein annexin A1 and its receptor ALXR (6, 80), as well as that of MerTK (73, 81). Long-term effects of glucocorticoids were reported to be mediated by PPARγ (82).

LXR agonists were shown to be effective in the treatment of mouse models of atherosclerosis and inflammation. Thus, LXR agonists [hypocholamide, T0901317, GW3965, or *N,N*-dimethyl-3β-hydroxy-cholenamide (DMHCA)] lower the serum cholesterol, and inhibit the development of atherosclerosis in murine models of atherosclerosis (83), while GW3965 inhibits the expression of inflammatory mediators in cultured macrophages as well as during *in vivo* inflammation (84). In addition, ligation of LXR was shown to prevent the development of SLE like autoimmunity in lpr mice (61) and decrease the disease severity in Alzheimer disease (85).

While all LXR ligands are effective in enhancing efferocytosis, T0901317, and GW3965 have been reported to increase plasma and liver triglycerides in some mouse models (86). DMHCA, however, reduced atherosclerosis in apolipoprotein E-deficient mice without inducing hypertriglyceridemia and liver steatosis (87). Thus, developing new potent and effective LXR agonists without the undesirable side effects may be beneficial for clinical usage (88). In this aspect, it is worth noting that we found daidzein, which is a plant-derived diphenolic isoflavone present in a number of plants and herbs (89) and has LXR and PPARγ activating activity (90), to enhance efferocytosis efficiently. Daidzein, similar to LXR agonists (91) induced the expression of TG2, as well as decreased the mitochondrial membrane potential (92).

In addition to LXR agonists, PPARγ agonists were also shown to reduce the neutrophil numbers in rodent models of acute inflammation, such as asthma and COPD (93) and to increase efferocytosis and therapeutic efficacy in a mouse model of chronic granulomatosis (94). PPARγ and PPARδ agonists were also shown to attenuate disease severity in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (95, 96).

Both RAR and RXR ligands promote efferocytosis, but their effect is more pronounced if both receptors are activated (63). The effect of *in vivo* all-*trans* retinoic acid (ATRA) treatment on the development of lupus nephritis has already been tested in both mouse models (97, 98) and humans (99). Lupus nephritis is a major cause of morbidity and mortality in patients with SLE (100). Long-term ATRA treatment in SLE-prone mice resulted in longer survival, significant reduction of proteinuria, renal pathological findings, and glomerular IgG deposits. In humans, it also reduced proteinuria.

### AFFECTING THE Rac-1/RhoA BALANCE

Since previous studies have shown that Rac activation is required, while RhoA activation is inhibitory for effective clearance of apoptotic cells (44), compounds that alter the Rac-1/RhoA balance, by either increasing the level of active Rac-1 or decreasing the levels and/or activity of RhoA/Rho kinase, would be potential candidates for use in therapy. Among the anti-inflammatory drugs glucocorticoids were shown to alter the Rac-1/RhoA balance in macrophages (101). Another molecule that was shown to affect the Rac-1/RhoA balance is lipoxin A4, which enhances phagocytosis via a protein kinase A-dependent manner (102). Though lipoxin A4 activates both Rac-2 and RhoA, its positive effect on efferocytosis suggests that the ultimate balance favors Rac activation. Lipoxins have already been shown to reduce inflammation and tissue damage in a variety of rodent models (103), and their levels are low in cystic fibrosis patients (104). In addition, exposure to daidzein also enhances Rac activity (92).

Statins are 3-hydroxy-3-methylglutaryl coenzyme A-reductase inhibitors with potent anti-inflammatory effects, largely due to their ability to inhibit the prenylation of Rho GTPases, including Rac-1 and RhoA. Since proper membrane localization of these proteins determines their function, statins inhibit the effectiveness of G protein signaling. Lovastatin was shown to enhance efferocytosis *in vitro* both in naïve murine lung and in alveolar macrophages taken from COPD patients (105). It was demonstrated that its effect is related to a disproportional deactivation of the RhoGTPases favoring the activity of Rac-1, as well as to the activation of PPARγ (106).

During inflammation oxidant-mediated activation of RhoA and inhibition of efferocytosis might be reversed by antioxidant treatment. Thus, in an LPS-induced lung injury model, antioxidants enhanced efferocytosis and reduced inflammation by inhibiting RhoA activation (107).

### AFFECTING PHAGOSOME MATURATION

Increasing evidence suggests that autophagy and phagocytosis processes are interactive and co-regulated. Thus, activation of autophagy during salivary gland cell death in the *Drosophila* requires the engulfment receptor Draper (108). In addition, association of LC3 with intracellular membranes described originally during autophagy was observed during phagocytosis as well (109). In line with these observations, oridonin, an active diterpenoid isolated from *Rabdosia rubesens*, was able to induce both autophagy and enhance efferocytosis in the human macrophage-like U937 cells. Moreover, enhancing autophagy by rapamycin also enhanced phagocytosis of apoptotic cells by U937 cells

(110). Thus, autophagy inducers might also promote efferocytosis. Though rapamycin and the so-called rapalogs are the most effective clinically used inducers of autophagy, they have severe immunosuppressive effects (111). That is why alternative, non-toxic autophagy inducers (such as rilmenidine or carbamazepine) are being characterized for their pharmacological profile in suitable preclinical models (112, 113). In addition, other non-toxic compounds, such as resveratrol and spermidine, are also being evaluated for their potential to induce autophagy *in vivo* (114, 115). These two latter compounds were shown to induce autophagy by distinct pathways converging on the acetylproteome (116). Resveratrol was suggested to mediate the cardioprotective effect of red wine (117), while spermidine was shown to prolong the life span of various organisms in an autophagy-dependent manner (114). Though the effect of the latter compounds on efferocytosis has not been tested yet, it is interesting to speculate whether enhanced efferocytosis contributes to their observed beneficial *in vivo* effects.

### ALTERING THE MEMBRANE LIPID COMPOSITION OF MACROPHAGES

Finally, studies on ob/ob and db/db mice indicate that in type 2 diabetes, obesity, or atherosclerosis impaired efferocytosis might be related to altered membrane lipid compositions of macrophages. In these cases, fish oil diet had a reversal effect (24). ω-3 fatty acids provided by fish oil are known substrates for the biosynthesis of pro-resolving mediators, such as resolvins, protectins, and maresin which, similar to glucocorticoids or opsonization of apoptotic cells by iC3b (69, 118), act as enhancers of efferocytosis as well as promote the formation of $CD11b^{low}$ macrophages (119).

## CONCLUDING REMARKS

Apoptotic cell death is an integral part of the cell turnover in many tissues. If, however, dead cells are not properly cleared, their content is released and induces tissue damage, as well as long-term inflammation. It is increasingly recognized that improper phagocytosis of apoptotic cells contributes to the establishment and progression of a number of human chronic inflammatory diseases. During the past decade, our knowledge about the mechanisms involved in efferocytosis increased significantly providing potential pharmacological targets through which the efficiency of apoptotic clearance could be increased. Since enhanced phagocytosis is coupled to an enhanced anti-inflammatory response, targeting efferocytosis might provide an additional possibility in the treatment of a numerous human chronic inflammatory diseases.

## ACKNOWLEDGMENTS

This study was supported by Hungarian grants from the National Research Fund OTKA (K104228, K83865, and NK105046) and the TÁMOP 4.2.2.A-11/1/KONV-2012-0023 "VÉD-ELEM" project. The project is implemented through the New Hungary Development Plan co-financed by the European Social Fund and the European Regional Development Fund.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 18 June 2014; accepted: 09 July 2014; published online: 01 August 2014.*
*Citation: Szondy Z, Garabuczi É, Joós G, Tsay GJ and Sarang Z (2014) Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications. Front. Immunol. **5**:354. doi: 10.3389/fimmu.2014.00354*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 23 December 2014
doi: 10.3389/fimmu.2014.00656

# The role of nucleotides and purinergic signaling in apoptotic cell clearance – implications for chronic inflammatory diseases

***Jin Chen, Yi Zhao* and Yi Liu***

*Department of Rheumatology and Immunology, West China Hospital, Sichuan University, Chengdu, Sichuan, China*

***Edited by:***
*Martin Herrmann, Universitätsklinikum Erlangen, Germany*

***Reviewed by:***
*Pablo Pelegrin, Fundacion Formacion Investigacion Sanitaria Region Murcia, Spain*
*Luis Enrique Munoz, Friedrich-Alexander University, Germany*

****Correspondence:***
*Yi Zhao, Department of Rheumatology and Immunology, West China Hospital, Sichuan University, 37# Guoxue Xiang, Chengdu, Sichuan 610041, China*
*e-mail: zhao.y1977@163.com*

Billions of cells undergo apoptosis every day in healthy individuals. A prompt removal of dying cells prevents the release of pro-inflammatory intracellular content and progress to secondary necrosis. Thus, inappropriate clearance of apoptotic cells provokes autoimmunity and has been associated with many chronic inflammatory diseases. Recent studies have suggested that extracellular adenosine 5′-triphosphate and related nucleotides play an important role in the apoptotic clearance process. Here, we review the current understanding of nucleotides and purinergic receptors in apoptotic cell clearance and the potential therapeutic targets of purinergic receptor subtypes in inflammatory conditions.

**Keywords: ATP, extracellular nucleotides, purinergic signaling, apoptotic cell clearance, chronic inflammation**

## INTRODUCTION

Apoptosis occurs in all multicellular organisms and plays a role in getting rid of superfluous and senescent cells during the development of an organism, tissue homeostasis, and pathogenic processes (1). In contrast to necrosis, apoptosis is a highly organized and fine-tuned process, and is, therefore, usually referred to as programed cell death. Besides the physiological process, apoptotic cells are also observed in tumors (2), atherosclerotic plaques (3, 4), and autoimmune diseases (5, 6). Under normal conditions, the apoptotic cell removal is performed very efficiently and fast by neighboring or recruited phagocytes and is important for maintaining the function of tissues (6, 7). Dying cells can undergo secondary necrosis if not cleared promptly and the release of intracellular contents has been linked to many human inflammatory diseases (8, 9). Moreover, apoptotic cells have been shown to have anti-inflammatory and regenerative effects (10).

Damaged tissues and dying cells can release nucleotides, which are increasingly viewed as a new class of regulators of the immune system. The class of purinergic receptors is involved in a wide range of phagocytic and chemotactic processes (11). Moreover, the purinergic signaling is an important regulatory mechanism in several inflammatory diseases (12). Several studies provide strong evidence that nucleotides and activated purinergic receptors are linked to the pathogenesis of many chronic inflammatory diseases. This review will discuss the apoptotic cell clearance with special emphasis the specific role of nucleotides and the purinergic receptors in the development of chronic inflammatory diseases related with abnormal clearance of apoptotic cells.

## COMPONENTS OF PURINERGIC SIGNALING

### EXTRACELLULAR ATP RELEASE AND METABOLISM

Damaged tissues and dying cells can release adenosine 5′-triphosphate (ATP) as a danger signal that triggers a variety of inflammatory responses. Moreover, ATP can also actively be released from intact cells in response to mechanical deformation, hypoxia or acetylcholine, which do not damage the cell (7, 13, 14). For example, ATP release from intact cells was firstly reported for neuronal cells, which release ATP into the cleft of chemical synapses (15). However, the underlying mechanism has been shown to be very complex and includes stretch-activated channels, voltage-dependent anion channels, P2X7 receptors, and connexin and pannexin hemichannels (16).

Contrasting to intracellular ATP, primarily utilized as energy, extracellular ATP is considered to be a powerful signaling molecule through the nucleotide-selective P2 receptors. Extracellular ATP is rapidly metabolized to adenosine by ectonucleotidases (17). The ectonucleotidases consist of four family types including (i) ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, (ii) ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, (iii) alkaline phosphatases (AP), and (iv) ecto-5′-nucleotidase (also known as CD73) (17, 18). Extracellular adenosine, an intermediate metabolite of nucleotides, can undergo three processes: (i) conversion to inosine by adenosine deaminase, (ii) reconversion to AMP by adenosine kinase, and (iii) cellular reuptake through concentrative nucleoside transporters (CNTs) or equilibrative nucleoside transporters (ENTs) (17, 19, 20).

### PURINERGIC RECEPTORS

Purinergic receptors have been widely studied in signaling systems in response to extracellular ATP and related nucleotides. Purinergic receptors consist of three major families based on their structural and biological properties (21). The G-protein-coupled P2Y receptors (P2YRs) recognize ATP and several other nucleotides, including ADP, UTP, UDP, and UDP-glucose (22). P2X receptors (P2XRs) function as ATP-gated ion channels that facilitate the influx and efflux of extracellular cations, including calcium ions, which only respond to ATP (22, 23). To date, P2YRs consist of eight subtypes, a family of P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14Rs. P2XRs have seven subunits that may form six homomeric (P2X1–P2X5Rs and P2X7R), and at least seven heteromeric P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, and P2X4/6Rs receptors (23–25). The conversion of ATP/ADP to adenosine by ectonucleotidases terminates P2R signaling within the extracellular compartment. Adenosine can signal through four distinct G-protein-coupled receptors (P1 receptors): adenosine A1 receptor (A1), adenosine A2a receptor (A2a), adenosine A2b receptor (A2b), and adenosine A3 receptor (A3) (**Table 1**) (26–28). The purinergic receptor subtypes are widely distributed throughout the immune cells and the central nervous system (CNS) (**Table 1**) (29–31).

## APOPTOTIC CELL RECOGNITION AND CLEARANCE

Apoptosis is a crucial process during development and regeneration of an organism. The prompt and efficient engulfment of apoptotic cells by phagocytes is necessary to prevent inflammation resulting from uncontrolled release of intracellular contents (34). Apoptotic cell clearance can be subdivided into four general steps: sensing of the apoptotic cell, recognition, engulfment of the corpse, and processing of the engulfed material (7, 35–38). Many key molecules and several molecular pathways have been identified to orchestrate the safe disposal of apoptotic cells. Apoptotic cells release so-called "find me" signals, which are cell-derived chemoattractants to entice phagocytes (9). To date, several proposed "find me" signals released by dying cells have been reported. These include the nucleotides ATP and UTP (39), lysophosphatidylcholine (LPC) (40), fractalkine (CX3CL1) (41), and sphingosine 1-phosphate (S1P) (42). In addition to attracting phagocytes, apoptotic cells are thought to release factors, referred to as "stay away" signals, to exclude inflammatory cells such as neutrophils (43).

At the same time, apoptotic cells also expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane as an "eat-me" signal to promote their recognition by the recruited phagocytes (44, 45). PS can be detected directly through membrane receptors, such as brain-specific angiogenesis inhibitor 1 (BAI1) (46),

**Table 1 | Characteristics of purinergic receptors [Modified from Ref. (15, 29, 32, 33)].**

| Receptor | | Distribution | Functions |
|---|---|---|---|
| P2Y | P2Y1 | Platelets, immune cells, epithelial and endothelial cells, and osteoclasts | Platelet aggregation, smooth muscle relaxation, and bone resorption |
| | P2Y2 | Astrocytes, immune cells, epithelial and endothelial cells, and osteoblasts | Promotes apoptotic cell removal; mediates airway surfactant secretion and epithelial cell chloride secretion; vasodilatation through endothelium and vasoconstriction through smooth muscle; bone remodeling; role in neutrophil chemotaxis; and chronic inflammation |
| | P2Y4 | Endothelial and epithelial cells | Epithelial chloride transport regulation; vasodilatation through endothelium |
| | P2Y6 | Activated microglia, T cells, and epithelial cells | Enhances microglial phagocytic capacity; modulating cytokines release; epithelium NaCl secretion; epithelial proliferation; and role in colitis |
| | P2Y11 | Dendritic cells, granulocytes | Mediates dendritic cells maturation and migration; granulocytic differentiation |
| | P2Y12 | Platelets and glial cells | Platelet aggregation; dense granule secretion |
| | P2Y13 | Spinal cord microglia, hepatocytes | Regulates lipid metabolism and atherosclerosis |
| | P2Y14 | Hematopoietic cells, immune cells | Hematopoietic stem cells chemotaxis; dendritic cell activation |
| P2X | P2X1 | Platelets, smooth muscle | Platelet activation; smooth muscle contraction |
| | P2X2 | Autonomic and sensory ganglia, retina | Sensory transmission and modulation of synaptic function |
| | P2X3 | Sensory neurons, sympathetic neurons | Mediates sensory transmission; facilitates glutamate release in CNS |
| | P2X4 | Microglial cell, immune cells | Modulates chronic inflammatory and neuropathic pain |
| | P2X5 | Dendritic cells | Mediating cell proliferation and differentiation |
| | P2X6 | Neuron, retina, and myocardial cell | Functions as a heteromeric channel in combination with P2X2 and P2X4 subunits |
| | P2X7 | Immune cells, osteoclasts, and microglia | Mediates apoptosis, cell proliferation and pro-inflammatory cytokine release |
| P1 | A1 | Neurons, autonomic nerve terminals | Modulates neurotransmitter release; treatment in cardiac tachycardia |
| | A2a | B cells, T cells | Anti-inflammatory effect; mediates cytokines release; facilitates neurotransmission; and smooth muscle relaxation |
| | A2b | Bronchial epithelial cells, cardiomyocytes | Dampens inflammation in allergic and inflammatory disorders; vasodilatation |
| | A3 | Endothelial cells, immune cells, and cardiomyocytes | Mediates anti-inflammatory, anti-ischemic, and antitumor effect |

stabilin 2 (47, 48), and members of the T cell immunoglobulin mucin domain (TIM) protein family (including TIM1, TIM3, and TIM4) (49–51). The recognition of apoptotic cells can also be mediated indirectly via bridging molecules or accessory receptors, such as MFG-E8, the C-reactive protein, and Gas-6 (52, 53). Engagement of the PS receptors initiates signaling events within the phagocytes that lead to activation of the small GTPase Rac, and subsequent cytoskeletal reorganization, which ultimately leads to engulfment of the apoptotic cell (54, 55).

The engulfment process is not only silent, but also actively anti-inflammatory. Firstly, phagocytes act as "garbage collectors," which sequester dying cells thus preventing the release of potentially dangerous or immunogenic intracellular contents. Secondly, engulfed phagocytes actively secrete anti-inflammatory cytokines to facilitate the "immunologically silent" clearance of apoptotic cells. These include TGF-β and interleukin (IL)-10, which is even potent enough to suppress LPS-induced inflammatory cytokine release (10, 56, 57). A recent report demonstrates that 12/15-lipoxygenase has been involved in maintaining immunologic tolerance (58). The uptake of apoptotic cells by 12/15-lipoxygenase expressing, alternatively activated resident macrophages blocked the uptake of apoptotic cells into freshly recruited inflammatory $Ly6^{Chi}$ monocytes. Moreover, loss of 12/15-lipoxygenase activity resulted in an aberrant phagocytosis of apoptotic cells by inflammatory monocytes, subsequent antigen presentation of apoptotic cell-derived antigens, and a lupus-like autoimmune disease (58).

If apoptotic cells are not removed promptly they will undergo secondary necrosis and display distinctive morphological changes that can be assessed by flow cytometry (59, 60). Insufficient clearance of dying cells may promote the initiation of autoimmunity and chronic inflammation (61, 62). For example, deregulated apoptosis and insufficient removal of apoptotic cells leads to the release of modified chromatin into the circulation and activation of antigen-presenting cells, which play an important role in the pathogenesis of systemic lupus erythematosus (61, 63). Interestingly, recent studies imply that apoptosis is associated with compensatory proliferation of neighboring cells and plays a pivotal role in modulating tumor cell repopulation (64, 65). For example, Huang et al. reported that dying tumor cells produce PGE2 in a caspase 3-dependent manner and that this has a potent growth-stimulating effect that may stimulate tumor repopulation after radiotherapy (66). The role of further "find me" signals and damage-associated molecular pattern molecules (DAMPs) released by tumor cells killed by chemo- or radiotherapy in the repopulation of the tumor remains elusive. Here, we present a current review that nucleotides derived from dead and dying cells as powerful mediators with broad effects on survival of tumor cells and on the immune system.

## NUCLEOTIDES ACTING AS "FIND ME" SIGNALS

It is well established that apoptotic cells release "find me" signals to attract phagocytes and thereby leading to the prompt clearance of the dying cells. The nucleotides ATP and UTP have been recently implicated as a new class of "find me" signals *in vitro* and *in vivo* (39). However, the function of ATP and nucleotides as a find me signal in apoptotic cell clearance is still controversial.

Elliot et al.'s study shows that small amounts of intracellular ATP and UTP are released in a regulated manner during early apoptosis to establish a gradient for monocyte attraction (39). Pannexin 1 channels opening mediate the release of ATP and UTP after caspase-dependent cleavage of their carboxy-terminal tail during apoptosis (67). Several other studies also seem to confirm that nucleotides released from apoptotic cells and subsequent P2Y2 receptor activation promotes monocyte migration by regulating adhesion molecule/chemokine expression in vascular endothelial cells (68, 69). In the neural system, extracellular nucleotides and P2YRs have been implicated in mediating the chemotaxis of microglia toward injured neurons (70, 71).

However, the role of nucleotides in chemotaxis still remains controversial. On the one hand, Elliot et al. could not exclude the possibility that other chemotactic factors participate in the observed chemoattractant effect. On the other hand, nucleotides are unlikely to serve as long-range "find me" signals to phagocytes since they are readily degraded by extracellular nucleotidases (72). Several recent publications do not consider ATP any longer as a "real" direct chemoattractant for macrophages. One study describes ATP as an indirect chemoattractant that steers macrophages in a gradient of the chemoattractant C5a via autocrine release of ATP, generating an amplification of gradient sensing via a "purinergic feedback loop" involving P2Y2 and P2Y12 receptors (73). Hanley et al. confirmed that ATP and ADP leaking from dying cells induce lamellipodial membrane protrusive activity and act as local short-range "touch me" (rather than long-range "find me") signals to promote phagocytic clearance (74). It is more likely that ATP, together with additional find me signals recruit phagocytes toward injured cells (75, 76). For example, formyl peptides and mitochondrial DNA released from the mitochondria of injured cells have been shown to induce neutrophil activation and chemotaxis in the circulation (77). Formyl peptides, together with chemokines and ATP, synergistically guide and localize phagocytes to sites of sterile inflammation in long-range settings (75). HMGB1 could also synergize with ATP stimulating P2X7 receptors to induce IL-1β release by DCs in contact with dying tumor cells and promoting immunity against tumors (78).

Moreover, nucleotides also play a role in modulating the phagocytic ability or activity of cells surrounding the apoptotic cells. For example, extracellular nucleotides and subsequent P2 receptor (P2X1R, P2X3R) signaling engagement have been reported to enhance the ability of macrophages to bind apoptotic bodies, internalize them and present processed antigens (79). UDP has also been shown to enhance microglia phagocytosis toward apoptotic corpses through the P2Y6 nucleotide receptor during neural inflammation (80).

During tissue injury and/or infection, extracellular nucleotides have been implicated to play a key role in the recruitment of professional phagocytes to sites of tissue injury and/or infection. However, the underlying mechanism is still unclear and not fully understood. It is still debating that extracellular nucleotides act either as chemotactic "find me" signal released by dying cells or through autocrine ATP amplifier signaling for chemotactic navigation to other end-target chemoattractants, such as complement C5a.

## P2 RECEPTORS SIGNALING IN INFLAMMATORY DISEASES

Nucleotides release from dying cells and damaged tissues and subsequent purinergic signaling play a pivotal role in phagocytic process and inflammatory diseases (11, 12). For example, P2X7R activation is involved in PS expose in pseudoapoptosis and large amounts of ATP release (81, 82). During the last decade, several studies have highlighted fundamental roles for P2YRs in inflammatory and infectious diseases (**Figure 1**). Here in particular, signaling events via P2Y2R, P2Y6R, and P2X7R will be discussed thoroughly.

### P2Y2R

P2Y2R has been shown to be up-regulated in a variety of tissues in response to stress or injury and to mediate tissue regeneration through its ability to activate multiple signaling pathways. Many studies implicate that ATP and P2Y2R signaling appears to influence a diverse scale of biological processes such as the generation of chemotactic signals and/or the activation of different immune cells, causing inflammatory cells to migrate, proliferate, differentiate, or release diverse inflammatory mediators (72, 83, 84).

Cystic fibrosis is a life-shortening disease in which airways of the patients are susceptible to infection. Its pathology is characterized by protective and also destructive neutrophilic inflammation. Neutrophil proteases are critical for killing engulfed bacteria, however, neutrophilic elastase accumulation in the airways of patients with cystic fibrosis (CF) overwhelms antiprotease defenses, resulting in impaired ciliary function, crippling bacterial clearance, and degrading structural proteins, eventually leading to bronchiectasis (85). CF results from a variety of mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, a cAMP-regulated chloride channel in epithelial cells, which will lead to sodium hyperabsorption in the airway of patients with CF (86, 87). Mucociliary clearance in CF lung is limited by airway dehydration, leading to persistent bacterial infection and inflammation. P2Y2 receptors have been shown to regulate chloride secretion and sodium absorption on epithelial cells in distal bronchi (88). Moreover, ATP, acting through P2Y2 receptors, regulates the secretion of ions, mucin, and surfactant phospholipids in respiratory epithelium (89). Several studies have shown that P2 receptor purinergic compounds are explored for the treatment of CF, to bypass the defective function of CFTR, and to restore chloride secretion and/or inhibit sodium absorption through inhibiting the epithelial sodium channel ENaC expression (90). P2Y2R agonists increase the duration of mucociliary clearance stimulation. The efficacy and safety of the P2Y2R agonist denufosol has been evaluated in several clinical trials, however, long term follow-up results do not show any improvement in pulmonary function (91, 92).

P2Y2R is not only involved in enhancing mucociliary clearance, but also plays a role in promoting wound healing (93). Damaged fibroblasts release ATP or UTP and activate P2Y2R to enhance the proliferation and migration of fibroblasts. Wound size in WT mice decreases significantly compared to P2Y2R$^{-/-}$ mice, and WT mice express proliferation marker Ki67 and extracellular matrix (ECM)-related proteins VEGF. It indicates that triggering of P2Y2R may be a potential therapeutic target to promote wound healing (94).

Adenosine 5′-triphosphate has also been implicated to induce chemotaxis of neutrophils via actin polymerization and direct cell orientation by feedback signaling involving P2Y2R (95–97). The subsequent P2Y2R activation will amplify gradient sensing of chemotactic signals (e.g., N-formyl peptides and IL-8) by stimulating F-actin to the leading edge (97–99). Chemotaxis of neutrophils to sites of infection is critical for immune defense

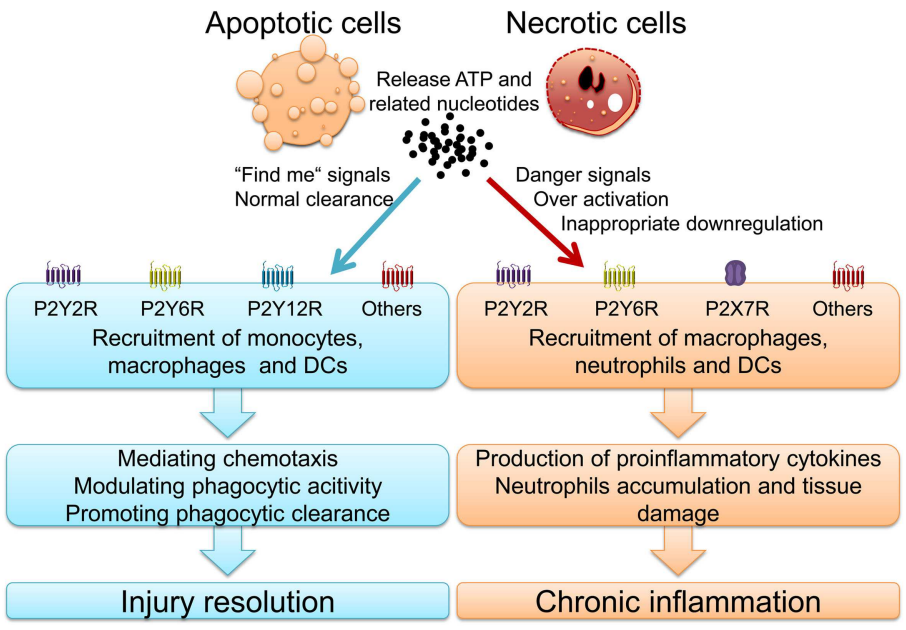


**FIGURE 1 | Nucleotides and activated purinergic signaling during injury resolution and chronic inflammatory diseases.**

and for the physiological downregulation of neutrophil-driven inflammation (100).

However, excessive accumulation of neutrophils through inappropriate activation of P2Y2R can cause acute tissue damage during sepsis, chronic obstructive pulmonary disease (COPD), and hepatitis (101–104). COPD is one of the most common inflammatory diseases and is associated with inflammation of the small airways, which results in airway obstruction, destruction of parenchyma, and development of emphysema (105). ATP and activation of P2Y2R contribute to smoke-induced lung inflammation and to the subsequent development of emphysema (104). ATP acts as a "danger signal" recruiting neutrophils to the lung and inducing inflammation. P2Y2R$^{-/-}$ mice show reduced pulmonary inflammation and less emphysema development after short-term smoke exposure. ATP enhances chemotaxis and elastase release in blood neutrophils from patients with COPD, compared to normal healthy subjects (103).

In asthmatic chronic airway inflammation, P2Y2R has been indicated as a critical sensor for airway exposure to airborne allergens by mediating ATP-triggered migration of immature monocyte-derived DCs and eosinophils in both, mice and humans (106, 107). This process is accompanied with the production of pro-allergic mediators (for example, IL-33, IL-8, eosinophil cationic protein) from different cellular sources (107, 108). Moreover, heightened expression and localization patterns of P2YR are associated with chronic pancreatic diseases (109).

In summary, ATP and P2Y2R signaling is a double-edged sword. On the one hand, it can protect against infections, promote wound healing and enhance mucociliary clearance. On the other hand, it can also lead to uncontrolled inflammation and promotion of chronic inflammatory disease states and fibrotic remodeling (**Figure 1**) (109). Indeed, P2Y2R may be a new target for therapy of COPD and P2Y2R antagonists could be useful drugs for chronic inflammatory diseases.

#### P2Y6R

Similar to P2Y2R, P2Y6R plays an ambivalent role in inflammatory diseases. The receptor is crucial for innate immune responses against bacterial infection (110). Many studies show that P2Y6R activation is involved in the release of chemokines from immune cells, such as monocytes, DCs, eosinophils, and recruiting monocytes/macrophages during inflammation or infection (24, 110–114).

In neurodegenerative diseases, microglia are engaged in the clearance of dead cells or dangerous debris, which is crucial for the maintenance of brain functions. Extracellular ATP regulates microglial motility dynamics in the intact brain, and its release from the damaged tissues mediates a rapid microglial response toward injury (71). Moreover, UTP and UDP released from injured neurons have been shown to enhance microglial phagocytic capacity for dying cells via activation of P2Y6R, serving as an "eat-me" signal for microglia. This signal is considered to be an important initiator of the clearance of dying cells or debris in the CNS (80).

However, P2Y6R signaling is relatively harmful in endothelial or epithelial inflammation (111, 115). The idiopathic inflammatory bowel diseases (IBD) comprise two types of chronic intestinal disorders: Crohn's disease and ulcerative colitis, which result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host (116). Up-regulation of P2Y2R and P2Y6R in intestinal epithelial cells has been reported in experimental colitis (115).

Similarly, P2Y6R plays an important role in acute and chronic allergic airway inflammation, and selective blocking of P2Y6R or P2Y6R deficiency in structural cells reduces symptoms of experimental asthma. Recently, P2Y6 receptors have not only been found to be up-regulated in murine atherosclerotic plaques, but also to play a key role in MSU-associated inflammatory diseases (117, 118).

Thus, P2Y6R activation plays a role in innate immunity against infection whereas P2Y6R over-activation can result in harmful immune responses and chronic inflammation such as atherosclerosis, COPD, and IBD (**Figure 1**).

#### P2X7R

P2X7R are predominantly expressed on immune cells such as mast cells, macrophages, microglia, and dendritic cells (119). Many evidences implicate the role of P2X7R against microbes during inflammation and immune response (120, 121). Indeed, P2X7R signaling plays a key role in immune responses against bacterial and parasitic infection. It has been reported that P2X7R signaling is involved in the elimination of intracellular microbes – such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Leishmania amazonensis* – either by contributing to killing of the pathogen or by inducing cell death of infected macrophages (121). P2X7R is also involved in fever development via PGE2 and IL-1β production (122).

The P2X7R is widely recognized to mediate the pro-inflammatory effects of extracellular ATP. However, recently one study revealed that P2X7 receptor also acts as one of the scavenger receptor involved in the recognition and removal of apoptotic cells in the absence of extracellular ATP and serum (123). The P2X7R has drawn particular attention as a potential drug target due to its broad involvement in inflammatory diseases (124).

In the CNS, P2X7R activation contributes to neuroinflammation through the release of pro-inflammatory cytokines, such as IL-1β and TNF-α (125, 126). It also activates MAP kinases and NF-κB, resulting in up-regulation of pro-inflammatory gene products, including COX-2 (127) and the P2Y2R (128). Alzheimer's disease (AD) is the most common form of dementia and more than 35 million people worldwide suffer from AD (129). The appearance of plaques consisting of extracellular β-amyloid peptide (Aβ) is a neuro-pathological feature of AD, which is surrounded by reactive microglial cells (129, 130). In P2X7R$^{-/-}$ mice, Aβ triggered increase of intracellular $Ca^{2+}$, ATP release, IL-1β secretion, and plasma membrane permeabilization in microglia (131). In fact, *in vivo* inhibition of P2X7R in mice transgenic for mutant human amyloid precursor protein (APP) indicated a significant decrease of the number of hippocampal amyloid plaques (132). Thus, the identification of extracellular ATP and P2X7R as key factors in Aβ-dependent microglia activation unveils a non-conventional mechanism in neuroinflammation and suggests new possible pharmacological targets.

Extracellular ATP and P2X7R signaling also contributes to the development of smoking-induced lung inflammation and emphysema. P2X7R$^{-/-}$ mice exhibit decreased inflammatory responses, including a reduction in pulmonary fibrosis in a mouse

model of lung inflammation (133). Inhibition of this receptor may be a new possible therapeutic target for the treatment of COPD (133, 134).

The purinergic P2X7R is associated with activation and release of IL-1 and IL-18, which is strongly implicated in the multiple inflammatory pathways involved in the pathogenesis of rheumatoid arthritis (RA) (135–139). P2X7R has also been shown to be expressed by synoviocytes from RA joints and contributes to modulation of IL-6 release (140). P2X7R activation also plays a novel and direct role in tissue damage through release of cathepsins in joint diseases (141). Although, AZD9056, a P2X7R antagonist, has been shown to reduce articular inflammation and erosive progression (142), clinical trials with the P2X7R antagonist in patients with RA failed to inhibit disease progression (143, 144). Similarly, the effect and safety of AZD9056 in Crohn's disease is still under clinical trial (145).

Taken together, P2X7R signaling not only plays a critical role in mediating appropriate inflammatory and immunological responses against invading pathogens, but also contributes to a wide range of chronic inflammatory diseases when activated inappropriately (**Figure 1**).

## CONCLUSION

The interaction between dying cells and phagocytes is very complex and nucleotides have been involved in orchestrating the process of dead cell removal. On the one hand, nucleotides and purinergic signaling have been shown to play a key role in the apoptotic cell clearance avoiding secondary necrosis, preventing inflammation and contributing to regeneration of injured tissues. On the other hand, purinergic signaling over-activation is involved in chronic inflammation and chronic inflammatory diseases. Adenosine-mediated P1 and nucleotides-mediated P2 signaling frequently have opposing effects in biological systems, and shifting the balance between P1 and P2 signaling is an important therapeutic concept in efforts to dampen pathological inflammation and promote healing (12). Nucleotides and purinergic signaling might be used as biomarkers for various diseases and could also provide potential novel therapeutic targets for the treatment of chronic inflammatory diseases.

## ACKNOWLEDGMENTS

The present work was supported by the National Science Foundation of China (81273286) to Dr. Yi Zhao.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 09 November 2014; accepted: 08 December 2014; published online: 23 December 2014.*
*Citation: Chen J, Zhao Y and Liu Y (2014) The role of nucleotides and purinergic signaling in apoptotic cell clearance – implications for chronic inflammatory diseases. Front. Immunol. **5**:656. doi: 10.3389/fimmu.2014.00656*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 01 July 2014
doi: 10.3389/fimmu.2014.00307

# Heat shock protein 70 serum levels differ significantly in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma

**Mathias Gehrmann[1], Melchiorre Cervello[2], Giuseppe Montalto[2,3], Francesco Cappello[4,5], Alessandro Gulino[6], Clemens Knape[1], Hanno M. Specht[1] and Gabriele Multhoff[1,7]***

[1] Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany
[2] Institute of Biomedicine and Molecular Immunology "Alberto Monroy," National Research Council, Palermo, Italy
[3] Biomedical Department of Internal Medicine and Specialties, University of Palermo, Palermo, Italy
[4] Section of Human Anatomy, Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Palermo, Italy
[5] Euro-Mediterranean Institute of Science and Technology, Palermo, Italy
[6] Tumor Immunology Unit, Department of Health Science, Human Pathology Section, School of Medicine, University of Palermo, Palermo, Italy
[7] Clinical Cooperation Group (CCG) – Innate Immunity in Tumor Biology, Helmholtz Centre Munich, German Research Centre for Environmental Health, Munich, Germany

**Edited by:**
Udo S. Gaipl, University Hosptial Erlangen, Germany

**Reviewed by:**
Alan Graham Pockley, Nottingham Trent University, UK
Luis Enrique Munoz, Friedrich-Alexander University, Germany

***Correspondence:**
Gabriele Multhoff, Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Ismaningerstr. 22, Munich 81675, Germany
e-mail: gabriele.multhoff@lrz.tu-muenchen.de

Members of the heat shock protein 70 (HSP70) family play an important role in assisting protein folding, preventing protein aggregation and transport of proteins across membranes under physiological conditions. Following environmental (i.e., irradiation, chemotherapy), physiological (i.e., cell growth, differentiation), and pathophysiological (i.e., inflammation, tumorigenesis) stress, the synthesis of heat shock proteins (HSPs) is highly up-regulated, whereas protein synthesis in general is reduced. In contrast to normal cells, many tumor entities including hepatocellular carcinoma (HCC) overexpress HSP70, the major-stress-inducible member of the HSP70 family, present it on their cell surface and secrete it into the extracellular milieu. Herein, the prognostic relevance of serum HSP70 levels in patients with chronic hepatitis (CH; $n = 50$), liver cirrhosis (LC; $n = 46$), and HCC ($n = 47$) was analyzed. Similar to other tumor entities, HSP70 is also present on the surface of primary HCC cells. The staining intensity of intracellular HSP70 in HCC tissue is stronger compared to control and cirrhotic liver sections. HSP70 serum levels in all HCC patients were significantly higher compared to a control group without liver disease ($n = 40$). No significant age- and gender-related differences in HSP70 serum levels were observed in male and female healthy human volunteers ($n = 86$). Patients with CH ($n = 50$) revealed significantly higher HSP70 serum levels compared to the control group, however, these values were significantly lower than those of HCC patients ($n = 47$). Furthermore, a subgroup of patients with LC who subsequently developed HCC (LC-HCC, $n = 13$) revealed higher HSP70 serum levels than patients with LC ($n = 46$, $p = 0.05$). These data indicate that serum HSP70 levels are consecutively increased in patients with CH, LC and liver carcinomas and thus might have a prognostic value.

**Keywords: HCC, serum HSP70, prognostic biomarker, chronic hepatitis, inflammation, liver cirrhosis**

## INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is increasing dramatically in the Western societies in the last years and HCC is the third leading cause of cancer-related deaths (1). Heavy alcohol intake, tobacco, vinyl chloride, and aflatoxin-B1 toxin can initiate HCC in humans. Apart from toxins, HCC can also arise from a dysregulated expression of small non-coding microRNAs (i.e., miR-122), diabetes, non-alcoholic fatty liver disease, hemochromatosis, liver cirrhosis (LC), and chronic hepatitis (CH) B/C viral infections. The exact molecular mechanisms that promote the transition of diseased liver cells into neoplastic lesions remain to be unsolved. The production of pro-inflammatory cytokines and chemokines, which induces a chronic inflammation in the liver are discussed to increase the risk for a malignant transformation (2–4). These data indicate that a multitude of different parameters including toxins, diseases, and the microenvironment of the host can play a role in the development of HCC (5).

At present, the histological evaluation of liver biopsies using the Edmondson–Steiner classification is the gold standard for the grading of HCC. For patients suffering from HCC with an underlying LC the Barcelona Clinic Liver Cancer group (BCLC) classification is used to describe the tumor volume, the grade of cirrhosis and the patient performance status. Apart from morphological inspections, antibodies directed against cyclase-associated protein 2 (CAP2) or glypican-3 are applied in immunohistochemistry to distinguish different tumor stages and to separate malignant from non-malignant lesions (6). Several other tumor biomarkers, such as p53, mammalian target of rapamycin (mTOR),

c-MET, insulin-like growth factor 1 receptor (IGF-1R), histone MacroH2A1 (7), and heat shock proteins (HSP) (8) including HSP70 are frequently up-regulated in tumor biopsies of HCC patients. However, the prognostic value of any of these markers alone is limited since its reliability can be impacted by gender and disease related parameters. Elevated mRNA levels of p53 are not only detected in male tumor patients with undifferentiated tumor stages but also in patients with cirrhosis (5). Similar results were found for an increased expression of mTOR that is also associated with other malignancies (5).

A major disadvantage of biopsy-based biomarkers is their limited availability and the risk to develop infections by the surgical intervention. Soluble, blood-derived biomarkers are superior to biopsies since they are easily accessible and can be taken repeatedly by using minimal invasive methods. In the present study, we aim to evaluate the prognostic significance of the major stress-inducible HSP70 in the serum of patients as a potential biomarker to distinguish patients with chronic inflammation (i.e., patients with CH) and LC from patients with HCC.

Members of the HSP70 family play a pivotal role in assisting protein folding, preventing protein aggregation and transport of proteins across membranes under physiological conditions. Following environmental (i.e., irradiation, chemotherapy, oxygen radicals), physiological (i.e., cell growth, differentiation), and pathophysiological (i.e., inflammation, tumor growth) stress, the synthesis of HSPs in general, but especially that of HSP70, is highly up-regulated, whereas that of other proteins is down-regulated. In contrast to normal cells, tumor cells frequently overexpress HSP70 in the cytosol (9), present it on their plasma membrane (10) and can actively secrete it in lipid vesicles such as exosomes (8, 11). The vesicular export of HSP70 in extracellular fluids was reported to stimulate effector mechanisms of the immune system (11, 12). Immunohistochemical analysis of tumor biopsies suggests that HSP70 in combination with other markers such as glutamine synthetase could serve as a putative diagnostic marker in HCC (6). Apart from HCC (13), an elevated expression of HSP70 was also found in patients with early-stage pancreatic cancer (14). High cytosolic HSP70 levels can promote tumor growth, prevent apoptotic cell death and thus are often associated resistance to therapy and poor prognosis in many different cancer types (8).

## MATERIALS AND METHODS

### HEALTHY HUMAN VOLUNTEERS AND PATIENTS

Eighty-six male and female healthy human volunteers (HEALTHY, $n = 86$) at different ages were enrolled into the study as well as patients suffering from CH (CH, $n = 50$), LC ($n = 46$), HCC (HCC, $n = 47$), and patients without a liver disease ($n = 40$) (**Table 2**). Approval was obtained by the Ethics Committees of the University Palermo, Italy, and the Klinikum rechts der Isar, Technische Universität München, Germany. All procedures were in accordance with the ethical standards of the responsible institutional and national committees on human experimentation and with the Helsinki Declaration of 1975 as revised in 2008.

Serum samples were collected from human patients with and without liver disease and healthy human volunteers at different ages. Blood samples of patients were taken after overnight fasting. After centrifugation (10 min, 750 × *g*, room temperature), part of the serum was used to assay the main parameters of liver function by routine methods. Serum aliquots of 100–500 µl were stored at −80°C for the measurements of HSP70. Sera were thawed only once for testing. Serological testing for anti-HCV was performed using a commercial third-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostic System, Raritan, NJ, USA), in accordance with the manufacturer's instructions. Serum levels of HCV-RNA were evaluated qualitatively by the Amplicor HCV test, version 2.0 (Roche Diagnostics, Basel, Switzerland) and quantitatively at baseline by the Cobas Monitor Test, version 2.0 (Roche Diagnostics). Markers of HBV were tested using the Abbott radioimmunoassay kit (Abbott Laboratories, Abott Park, IL, USA).

### TUMOR BIOPSIES

Liver biopsy samples were obtained percutaneously according to the Menghini technique using needles of $1.0 \pm 1.2$ mm diameter (Surecut, Hospital Service, Rome, Italy). In some cases, HCC was diagnosed using a thin needle (20 Gage, Surecut) under ultrascan control, using a Toshiba SSA 240A apparatus with a 3.5-MHz probe. Tissues from HCC and adjacent liver were obtained from patients undergoing surgical resection. Histologically normal liver tissue was obtained from patients during surgery for cholelithiasis. Written informed consent was obtained in all cases; the protocol was approved by the local Ethics Committee 1(see above). Biopsies in the size range of a few mm were taken during tumor excision.

### FLOW CYTOMETRY

Single cells from freshly isolated tumor biopsies were prepared by mechanical disruption, as described previously (15). $1 \times 10^5$ cells were washed once with 10% FCS in phosphate buffered saline (PBS) and incubated with a FITC-conjugated mouse monoclonal antibody specific for membrane-bound HSP70 (cmHSP70.1, IgG1, multimmune GmbH, Munich, Germany) (16) or a FITC-labeled isotype-matched IgG1 negative control antibody (345815, BD Biosciences, Franklin Lakes, NJ, USA) on ice in the dark for 30 min. After washing, propidium iodide was added and viable cells were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). The percentage of cells stained with an isotype-matched control antibody was subtracted from the percentage of cmHSP70.1 antibody positive cells.

### IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical investigation was performed on specimens fixed in formalin and embedded in paraffin. Four micrometer-thick sections were cut, dewaxed, and hydrated, heated in a microwave oven (three to four cycles of 5 min each) in 10 mM citrate buffer (pH 6.0), then washed twice with PBS for 5 min. All sections were incubated in 3% hydrogen peroxide (v/v) in methanol for 5 min. Immunohistochemistry was performed with the Streptavidin–biotin complex (StreptABC) using rabbit polyclonal antibody against HSP70 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at a dilution of 1:200 for 2 h at 37°C. Sections were then incubated for 30 min at RT with biotinylated anti-rabbit immunoglobulin diluted in PBS, with StreptABC for 30 min at RT, and the color was developed with 3-amino-9-ethylcarbazole (AEC) (Dako, Copenhagen, Denmark) for 5–10 min at RT, and counterstained with Mayer hematoxylin for 3 min. Results

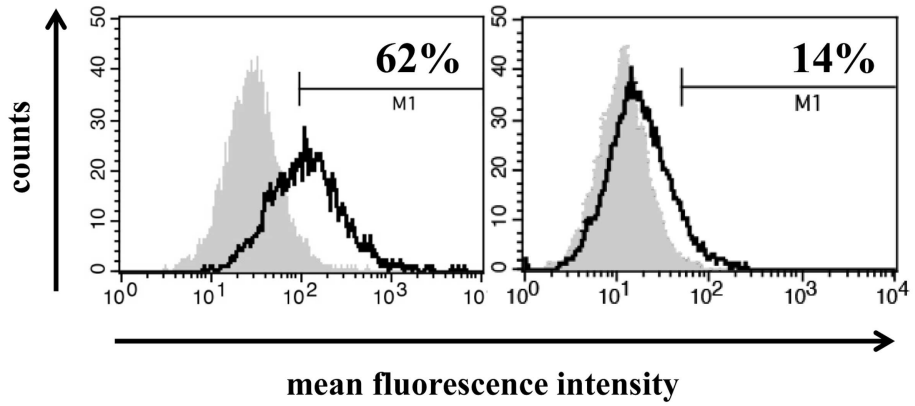


**FIGURE 1 | Representative images of a high (left) and low (right) HSP70 membrane expression on primary HCC cells**. Single cell suspensions from freshly isolated HCC biopsies derived from two different patients were incubated with FITC-conjugated mouse monoclonal antibody cmHSP70.1 that recognizes the membrane-bound form of HSP70 on the surface of tumor cells. The white histogram represents the HSP70 membrane staining of the tumor cells and the gray histogram the staining with a negative control FITC-conjugated isotype-matched control antibody. The percentage of HSP70 membrane-positive cells was corrected by subtraction of the isotype control.

were assessed semiquantitatively in blind by three expert pathologists and by counting the proportion of positively stained cells in 10 random high power fields at a 10 and 40× magnification.

### ELISA ASSAYS

Total HSP70 levels in serum samples of humans were measured using an HSP70 immunoassay (Duoset, DYC1663, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions with modified buffers. The ELISA is designed to detect inducible human HSP70. All serum samples were tested in three independent ELISA experiments in duplicates. HSP70 was detected by incubation with HRP-conjugated anti-human Ig followed by HRP-substrate staining (DY999, R&D Systems, Minneapolis, MN, USA). Signals were determined by measuring the absorption at 450 nm in a standard ELISA reader (BioTek, Winooski, VT, USA) with a correction wavelength set to 540 nm.

### STATISTICAL ANALYSIS

Statistical analysis was performed using SigmaPlot software delivered by Systat Software, Inc. (San Jose, CA, USA). Results of the levels of soluble HSP70 are presented as standard box plots with boundaries indicating the 25th and the 75th percentile. The line inside boxes indicates the median and the whiskers indicate the 10th and 90th percentile, respectively. All outliers are shown. For comparison between groups of data the Student's *t*-test or the Mann–Whitney Rank Sum Test were used to evaluate differences. *p*-Values $<0.05$ are considered to be statistically significant.

## RESULTS

### HSP70 MEMBRANE PHENOTYPE ON BIOPSIES OF PATIENTS WITH HCC

Freshly isolated, non-fixed biopsies of patients with HCC were minced and filtered through a sterile mesh to obtain single cell suspensions of the tumor. Directly after washing tumor cells were centrifuged and incubated with FITC-conjugated mouse monoclonal antibody (mAb) cmHSP70.1 that recognizes the membrane-bound form of HSP70 on the surface of viable tumor cells with an intact membrane (**Figure 1**, white histograms). As a negative control, a FITC-conjugated isotype-matched control antibody was used (**Figure 1**, gray histograms). The percentage of HSP70 positively stained cells was corrected according to the results from the staining with an isotype-matched control antibody. The histograms depicted in **Figure 1** show a typical result of a high (62%) and low (14%) membrane HSP70 positive tumor biopsy. Previous studies with biopsies of normal tissues revealed that a sample is considered as HSP70 membrane-positive if more than 20% of the cells are stained positively with the cmHSP70.1 mAb (17). With respect to this threshold, three out of five selected HCC patient samples were HSP70 membrane-positive (**Table 1**). This finding is in line with results derived from other human tumor entities ($n = 978$), showing that more than 50% of all tested tumor samples were found to be HSP70 membrane-positive (10, 15).

**Table 1 | HSP70 membrane status in single cell suspensions of HCC tissue obtained from tumor patients**.

| Patient no. | HSP70$^+$ cells (%) | Mean fluorescence intensity (MFI) | HSP70 phenotype |
|---|---|---|---|
| 1 | 62 | 429 | + |
| 2 | 95 | 1923 | + |
| 3 | 33 | 158 | + |
| 4 | 12 | 27 | – |
| 5 | 14 | 37 | – |

### REPRESENTATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF THE HSP70 PROTEIN CONTENT IN LIVER SECTIONS OF HUMAN VOLUNTEERS WITHOUT LIVER DISEASE (CTRL) AND HCC PATIENTS WITH UNDERLYING LC

The patient cohort consists of 183 subjects that could be divided into four groups (**Table 2**). Group 1 was composed of 40 patients (CTRL) without liver disease derived from the Biomedical

**Table 2 | Clinical and pathological features of control patients without liver diseases (CTRL) and patients with chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC); data are expressed as the median (range)**.

| Characteristics | Group 1 CTRL | Group 2 CH | Group 3 LC | Group 4 HCC |
|---|---|---|---|---|
| Number (*n*) | 40 | 50 | 46 | 47 |
| Gender (M/F) | 36/4 | 30/20 | 24/22 | 27/20 |
| Age (years) | 44 (23–63) | 52.5 (25–85) | 66.5 (30–86) | 73 (45–87) |
| Albumin (g/dl) | 4.7 (3.47–5.01) | 4.66 (4.0–4.9) | 3.4 (2.0–4.5) | 1.03 (0.24–5.56) |
| Bilirubin (mg/dl) total | 0.72 (0.52–1.0) | 0.75 (0.34–1.1) | 1.27 (0.15–5.89) | 1.03 (0.24–5.56) |
| Aspartate amino-transferase (IU/ml) | 18.7 (12.0–26.1) | 55 (25.0–173.0) | 70 (19.0–377.0) | 56 (12.0–204.0) |
| Alanine amino-transferase (IU/ml) | 16.2 (11.5–22.02) | 85 (31.0–251.0) | 57 (12.0–221.0) | 45 (12.0–230.0) |
| International normalized ratio (INR) | 0.92 (0.86–1.01) | 0.97 (0.91–1.07) | 1.24 (1.06–1.73) | 1.06 (0.82–1.75) |
| HBs Ag | – | 2 | 4 | 3 |
| HCV Ab | – | 38 | 32 | 34 |
| Alcoholism | – | None | 3 | 5 |
| Cryptogenic | – | 10 | 7 | 4 |
| Dysmetabolic | – | None | None | 1 |
| **BCLC** | | | | |
| A | – | – | – | 21 |
| B | – | – | – | 9 |
| C | – | – | – | 8 |
| D | – | – | – | 5 |
| E | – | – | – | 4 |
| **CHILD-PUGH** | | | | |
| A | – | – | 26 | – |
| B | – | – | 16 | – |
| C | – | – | 4 | – |

*BCLC, Barcelona Clinic Liver Cancer group; HBs Ag, anti-hepatitis B surface antigen; HCV Ab, anti-hepatitis antibody.*

Department of Internal Medicine and Specialties, University of Palermo, Palermo, Italy. Liver disease was excluded on the basis of anamnestic, biochemical, and instrumental data. In this group, no case of neoplastic disease was detected within a follow-up period of at least 6 months. Group 2 included 50 patients with CH infections and Group 3 included 46 patients with LC. Diagnosis was made on the basis of liver biopsies and unequivocal biochemical and instrumental data. The absence of neoplasia had been verified during a post-study follow-up period of at least 6 months. Finally, group 4 included 47 patients with HCC. Diagnosis was based on histology, cytology, multiple concordant imaging techniques [ultrasound, basal and lipiodol computed tomography (CT), selective angiography], and biochemical assays (serum levels of AFP $>200$ ng/ml). Some of the patients were known as liver cirrhotics and had been enrolled in a prospective study for HCC screening.

Representative immunohistochemical images were taken from sections of liver biopsies of human patients without liver disease (CTRL) and a patient who was diagnosed with HCC and LC (**Figure 2A**). The HSP70 staining intensity was stronger in the HCC tissue compared to that of the control liver (CTRL) and to the cirrhotic part (LC) of the patient biopsy. These data indicate that the intracellular HSP70 content is higher in the cancerous compared to the cirrhotic liver tissue. A comparison of four different LC-HCC patients revealed different staining intensities in the HCC and LC regions and between the different patient sections (**Figure 2B**).

### HSP70 PROTEIN LEVELS IN THE SERUM OF HUMAN VOLUNTEERS (HEALTHY), PATIENTS WITHOUT LIVER DISEASE (CTRL), AND PATIENTS WITH CHRONIC HEPATITIS, LIVER CIRRHOSIS, AND HEPATOCELLULAR CARCINOMAS

The soluble HSP70 levels were determined in the serum of 86 male (54) and female (32) healthy human volunteers at different ages. Irrespectively of the age and gender, the HSP70 serum levels did not differ significantly in the healthy human volunteers (HEALTHY) (**Figure 3A**). A comparison of the HSP70 levels in patients without liver disease (CTRL, $n = 40$, $2.7 \pm 0.9$ ng/ml) and healthy human volunteers (HEALTHY, $n = 86$, $2.3 \pm 0.8$ ng/ml) also revealed no significant differences (**Figure 3B**). In contrast, the HSP70 serum levels of patients with liver diseases such as CH, LC, and HCC differed significantly from that of healthy human volunteers and patients without liver disease. The highest serum HSP70 levels in patients were found in HCC (HCC, $n = 47$, $6.5 \pm 3.1$ ng/ml) and LC patients (LC, $n = 46$, $6.6 \pm 5.2$ ng/ml). The lowest HSP70 levels were found in patients with CH ($n = 50$, $3.9 \pm 2.4$ ng/ml). These values were significantly lower than that of HCC and LC patients (**Figure 3B**).

In order to evaluate whether the HSP70 serum levels could predict the risk to develop HCC a subgroup analysis was performed. As shown in **Figure 3C**, a small subgroup of HCC patients with underlying LC revealed higher HSP70 serum levels (LC-HCC, $n = 13$, $7.3 \pm 2.2$ ng/ml) than the overall group of patients with LC ($6.6 \pm 5.2$ ng/ml) with unknown HCC status. However, due to

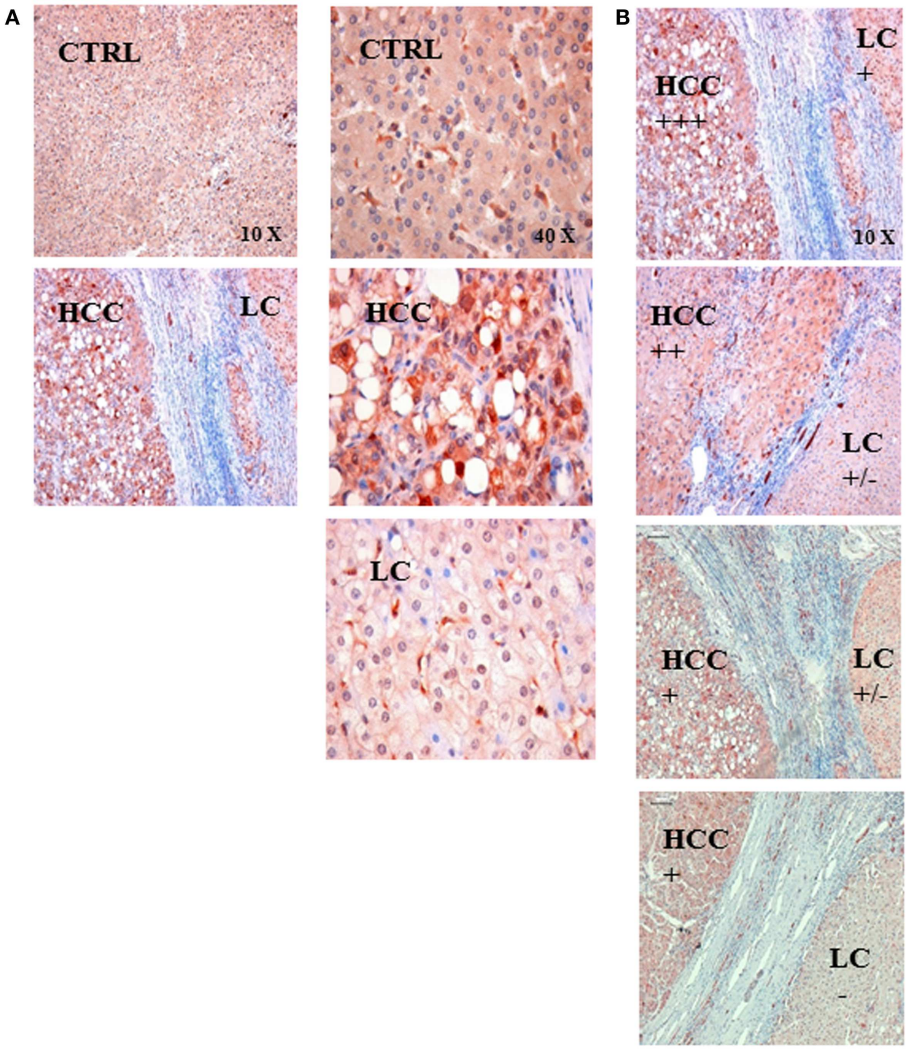


**FIGURE 2 | (A)** Representative immunohistochemical images of the HSP70 staining in control liver tissue (CTRL) and in the tissue of a patient with hepatocellular carcinoma (HCC) with an underlying liver cirrhosis (LC). The HSP70 staining intensity was stronger in HCC tissue compared to that of control liver tissue (CTRL) and in areas with liver cirrhosis (LC); left panel 10× magnification, right panel 40× magnification. **(B)** Semiquantitative analysis of the HSP70 staining intensity in sections of LC-HCC patients ($n = 4$) at a 10× magnification. The HSP70 staining intensity in the HCC regions ranged from very strong (+++), via intermediate (++) to strong (+); in the LC regions the staining intensity ranged between strong (+), weak (±), and very weak (−) in the four different sections.

the low number of patients ($n = 13$) only a trend ($p = 0.05$) was determined.

## DISCUSSION

Biomarkers are used to detect tumors, monitor tumor growth, and to assess the effectiveness of anti-cancer therapies (18). A major disadvantage of biopsy-based markers is the risk for developing infections caused by the invasive intervention. Since blood samples can be taken by minimal invasive methods from patients before, during, and after therapy this method is superior for tumor detection and for monitoring the clinical outcome. In this study, soluble HSP70 was examined for its potential prognostic significance to serve as a blood-derived biomarker to detect HCC and to distinguish HCC from other liver diseases such as CH and LC. Previous studies of our group already have shown that HSP70 membrane-positive tumors actively secrete HSP70 into the extracellular milieu in cell cultures (11). This result could be confirmed in tumor bearing mice (19) and in patients with squamous cell carcinomas of the head and neck (Ms submitted). Since the availability of tumor biopsy material is limited during the course of disease, we addressed the question whether serum HSP70 levels could reflect the HSP70 membrane status of the tumor cells also in HCC patients. Comparative analysis revealed that an increased intracellular HSP70 staining intensity of HCC cells in sections was associated with increased serum HSP70 levels in a selected group of patients who suffered from HCC and LC (data not shown). In a group of patients with HCC only, the cytosolic HSP70 levels did not correlate with soluble HSP70 levels. This is in line with the

findings of Kang et al. (5) who showed no correlation of cytosolic HSP70 levels with prognosis of HCC after resection. The excellent accessibility of serum biomarkers allows repeated testing during the course of a disease and the monitoring of clinical outcome. Serum HSP70 levels have been discussed to provide a useful biomarker for testing the efficacy of an Hsp90 inhibitor-based tumor therapy that is known to induce the expression of HSP70 (20).

It has been reported that HSP70 can be actively released by viable tumor cells with an intact cell membrane (21). In this study, we could show that patients with HCC exhibited significantly higher HSP70 serum levels compared to patients with hepatic viral infections (**Figure 3**). These findings might provide a hint that the largest proportion of soluble HSP70 in the serum is produced by viable tumor cells that actively secrete HSP70 in lipid vesicles and not by necrosis of inflamed liver tissue. Together with the finding that serum HSP70 levels correlate with the volume of viable tumor cells in mice (19), we hypothesize that soluble HSP70 levels might be useful to evaluate the mass of vital tumor cells in human patients before and after therapeutic intervention.

Since membrane HSP70 is frequently present on a broad variety of different tumor entities such as colorectal, lung, pancreatic, and prostate cancer patients (14, 20, 22, 23) and since membrane HSP70 positive tumor cells do secrete HSP70 into the extracellular milieu it is expected that soluble HSP70 levels might serve as a useful biomarker for different tumor entities. Elevated HSP70 serum levels have been found in cardiovascular, inflammatory and pregnancy-related diseases. In this study, we could show quantitative differences in soluble HSP70 levels in inflammation, cirrhosis, and cancer. Since the highest amount of HSP70 is actively secreted by tumor cells and not from inflamed and virally infected tissues, soluble HSP70 levels might provide a measure to determine the mass of viable tumor cells in patients (24).

## CONCLUSION

In the present study, the prognostic value of extracellular HSP70 was determined in the serum of patients with liver diseases such as CH, LC, and HCC. HSP70 serum levels were found to be significantly higher in cancer patients compared to healthy individuals, patients without liver diseases and patients with an inflammation of the liver. Our data encourage us to hypothesize that serum HSP70 might be a useful biomarker to differentiate HCC from other liver diseases.

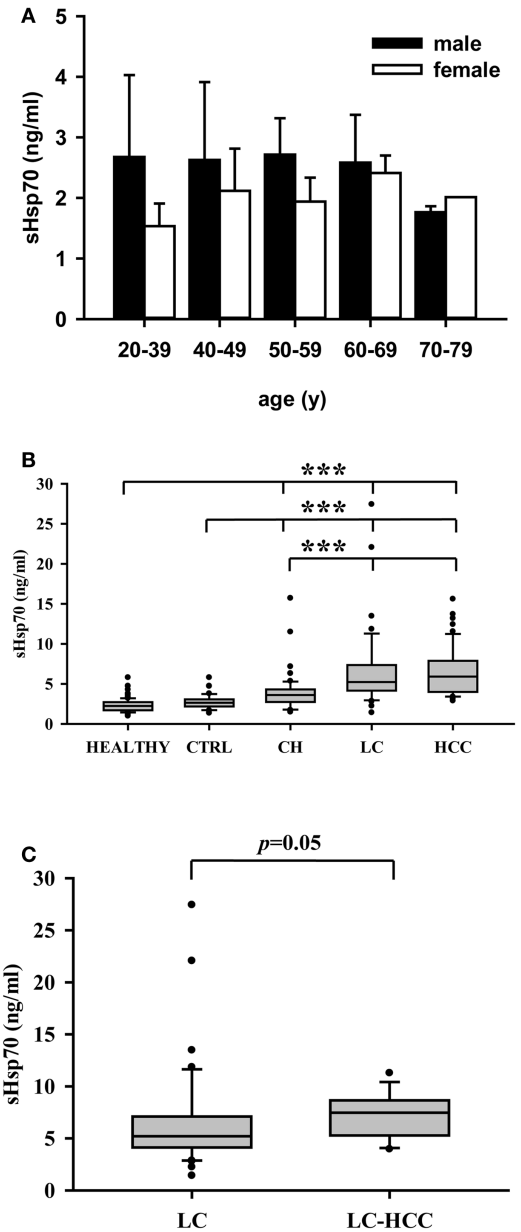


**FIGURE 3 | (A)** HSP70 protein levels in the serum of male ($n = 54$) and female ($n = 32$) healthy human volunteers at different ages ($n = 86$). The HSP70 serum levels did neither differ significantly in male and female healthy human individuals nor in different age groups ranging from 20–39, 40–49, 50–59, 60–69, to 70–79. **(B)** HSP70 protein levels in healthy human volunteers (HEALTHY, $n = 86$), healthy controls without liver diseases (CTRL, $n = 40$), patients with chronic hepatitis (CH, $n = 50$), liver cirrhosis (LC, $n = 46$), and hepatocellular carcinomas (HCC, $n = 47$). Serum HSP70 levels did not differ significantly between healthy human volunteers and controls without liver diseases, but both groups differed significantly to patients with CH, LC, and HCC. **(C)** HSP70 protein levels in the serum of patients with liver cirrhosis (LC, $n = 46$) and of HCC patients with an underlying LC (LC–HCC, $n = 13$). Serum HSP70 levels were higher ($p = 0.05$) in patients with HCC and an underlying LC compared to patients with LC only. Serum HSP70 levels were determined by sandwich ELISA. Values were determined at least three times in duplicates. Median values are shown as box plots. Significance was calculated by using the Mann–Whitney-*U*-test (***$p < 0.001$).

## ACKNOWLEDGMENTS

The authors want to thank Dr. Daniele Balasus for collecting patient's clinicopathological data and Jessica Pelzel for excellent technical assistance. This study was supported in parts by IEMST (Francesco Cappello), FIRB-MERIT no. RBENE08YYBM from the Italian Ministry for Education, the University and Research (MIUR) (Melchiorre Cervello, Guiseppe Montalto), CNR from the Italian Ministry of Economy and Finance for the Project FaReBio di Qualità (Melchiorre Cervello), the DFG – Cluster of Excellence: Munich-Centre for Advanced Photonics (Gabriele Multhoff), the DFG project SFB824/B4 (Gabriele Multhoff), the m4 – Cluster of Excellence: Personalized Medicine (BMBF, 16EX1021C) (Gabriele Multhoff), the BMBF "Innovative therapies" and "NSCLC" (BMBF, 01GU0823; 16GW0030) (Gabriele Multhoff), the Wilhelm Sander

Stiftung (2012.078.1) (Gabriele Multhoff) and EU-CELLEUROPE (EU315963) (Gabriele Multhoff).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 07 May 2014; accepted: 17 June 2014; published online: 01 July 2014.*
*Citation: Gehrmann M, Cervello M, Montalto G, Cappello F, Gulino A, Knape C, Specht HM and Multhoff G (2014) Heat shock protein 70 serum levels differ significantly in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Front. Immunol. 5:307. doi: 10.3389/fimmu.2014.00307*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*

frontiers in
IMMUNOLOGY

OPINION ARTICLE
published: 04 July 2014
doi: 10.3389/fimmu.2014.00299

# Sinister self-sacrifice: the contribution of apoptosis to malignancy

***Jorine J. L. P. Willems, Benjamin P. Arnold and Christopher D. Gregory****

*MRC Centre for Inflammation Research, Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, UK*
**Correspondence: chris.gregory@ed.ac.uk*

***Edited by:***
*Christian Berens, Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany*

***Reviewed by:***
*Patrizia Rovere Querini, Ospedale San Raffaele and Vita-Salute University, Italy*
*Lidija Klampfer, Montefiore Medical Center, USA; Albert Einstein Cancer Center, USA*

**Keywords: apoptosis, inflammation, lactoferrin, phagocytosis, tumor-associated macrophages**

Induction of apoptosis is one of the main defenses of the body against cells that have acquired malicious mutations. It may seem counter-intuitive then, that massive cell death is observed in many malignant tumors (1, 2). Despite high rates of apoptosis, these tumors continue to grow rapidly. Thus, tumor cell growth must outbalance tumor cell death. Intuitively presumed only to inhibit tumor growth, apoptotic cells may actually promote net tumor growth (3, 4). As long ago as 1956, Revesz showed that cell death can enhance tumor growth (5). Moreover, new studies in progress in our laboratory show that apoptosis in tumor cells promotes growth rates in aggressive B-cell lymphoma.

Cells undergoing apoptosis are difficult to observe *in vivo*, as they are rapidly cleared by phagocytosis, most obviously by macrophages. Accumulation of macrophages, sometimes engorged with apoptotic cells, is observed in many malignant tumors and is generally associated with poor prognosis (6, 7). Inflammatory cells, in particular macrophages, are key elements of the tumor environment, providing support for the continually expanding "rogue" tissue. The tumor microenvironment resembles that of a wound that fails to heal (8), where macrophages not only clear and repair, but also promote tissue regeneration and support. Tumor-associated macrophages (TAM) display a phenotype that is reminiscent of wound-healing macrophages. They have been shown to promote angiogenesis, tissue remodeling, and anti-inflammatory responses, which results in the support of tumor cell growth and metastasis (9–12).

Apoptosis of tumor cells in a growing malignant tissue may therefore be rationalized as a "sinister sacrifice" of some cancer cells that ultimately facilitates cancer progression. We hypothesize that apoptotic cells play a key role in driving oncogenesis, both through the release of soluble and microvesicle-associated signaling factors, as well as through direct interaction with phagocytes. Here we postulate lactoferrin (Lf) as an important signaling factor maintaining an anti-inflammatory tumor microenvironment, and stress the importance of apoptotic cell engulfment by macrophages for driving a pro-tumor phenotype in TAM (**Figure 1**).

## DIRECT EFFECTS OF LACTOFERRIN

Cells undergoing apoptosis release a variety of biologically active "find-me" or "keep-out" signaling factors, including the nucleotides ATP and UTP (13, 14), the lipid lysophosphatidylcholine (LPC) (15), as well as the proteins fractalkine (16), Lf (17), and monocyte chemotactic protein (MCP-1) (18). Some of these signaling molecules may be associated with microvesicles, as is the case with the chemokine, fractalkine (16), which may support prolonged biological activity. It has been hypothesized that these find-me signals not only affect chemotaxis and phagocytosis, but may also have additional pleiotropic biological effects. Indeed, ATP released by apoptotic cells increased binding of apoptotic cells to macrophages (19). Furthermore, fractalkine has been shown to stimulate pro-survival and growth-promoting effects (20, 21) and was found to cause the expression of milk fat globule epidermal growth factor (MFG-E8) on macrophages, which leads to enhanced apoptotic cell clearance (22).

Lactoferrin was identified in our laboratory to be released from apoptotic cells. Lf is produced *de novo* by a diverse range of cells stimulated to undergo apoptosis *in vitro* (17). This 80 kDa iron-binding glycoprotein is well-documented to have immunomodulatory, antimicrobial, anti-inflammatory, and trophic activities (23–26). We propose that Lf is another pleiotropic molecule released from apoptotic cells that can regulate the tumor microenvironment. Thus, since it is well-known that apoptosis is frequent in several types of cancer, particularly high-grade forms (27), it is conceivable that persistence of uncleared apoptotic cells (which may occur through saturation) could enable these cells to become secondarily necrotic with the potential consequences of release of noxious contents via cell lysis leading to activation of pro-inflammatory responses (28). However, most malignant tumors maintain a phenotype that militates against anti-tumor immune and inflammatory responses. Given our previous findings that Lf is released from cells undergoing apoptosis (17), together with our unpublished studies showing that Lf binds to necrotic cells, we suggest that Lf serves to dampen down pro-inflammatory responses resulting from persistent secondarily necrotic cells. In fact, it has been shown that necrotic neutrophil lysates, which contain large quantities of Lf from the secondary granules, are anti-inflammatory, and are able to inhibit the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), IL6, IL8, and IL1β, by macrophages (29). The mechanism through which this is achieved may involve the "mopping up" of necrotic cell-released pro-inflammatory contents by Lf.

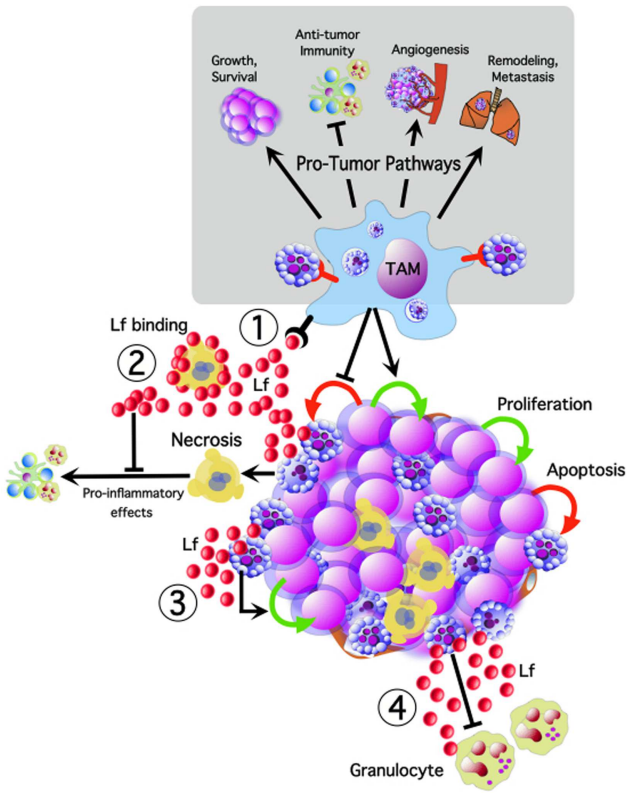


**FIGURE 1 | Tumor cell apoptosis potentially activates multiple oncogenic pathways, promoting tumor cell growth and survival, angiogenesis, remodeling, and metastasis, while inhibiting anti-tumor immune responses**. We propose that TAM interacting with apoptotic tumor cells are central to many of these pathways. Apoptotic cells release lactoferrin (Lf) which could promote tumor growth and progression by: (1) TAM activation, (2) modulating the inflammatory effects of necrosis, (3) acting as a direct trophic factor, and (4) functioning as a "keep-out" signal to anti-tumor granulocytes.

Lactoferrin has a highly positively charged N-terminal end (30), which is capable of interacting with a variety of proteins and membranes, but can also bind a selection of metal ions as well as iron (31). Furthermore, Lf can interact with lipid A of lipopolysaccharide (LPS) causing the neutralization of LPS-stimulated secretion of pro-inflammatory cytokines by monocytic cells, including TNFα, IL1β, IL6, and IL8 (32, 33). Mopping of noxious contents by Lf may be a final safeguard system to prevent pro-inflammatory responses at sites of high rates of apoptosis. This may not only help maintain the anti-inflammatory environment in tumors, but could also play a role in the resolution of inflammation, where neutrophil activation and death may lead to the release of large quantities of Lf. In addition, in tumors characterized by neutrophil infiltration, the dominant source of biologically active Lf may be derived from neutrophils, rather than apoptotic tumor cells.

Lactoferrin is also known to directly exert anti-inflammatory effects by inhibiting the migration of neutrophils (17) and also by indirectly enhancing the production of anti-inflammatory cytokines including IL4, IL10, and transforming growth factor-β (TGFβ) (25, 26). Some studies also suggest that Lf can directly interact in the nuclear factor κB (NFκB) pathway interfering with its binding to DNA (33).

These findings point to a possible direct mechanism of Lf for controlling pro- and anti-inflammatory cytokine expression. In high-grade malignancies, these effects of Lf could help moderate anti-tumor inflammatory and immune responses, allowing continued malignant growth. The pro-tumor effects of Lf are likely to be context dependent, however, since Lf has been shown to have pro-inflammatory, immunostimulatory, and cell growth-inhibitory effects (34–36) as well as anti-inflammatory and trophic properties. An open, and important question is whether Lf is released by dying tumor cells as a consequence of anti-tumor therapy and, if so, whether it has properties which could ultimately confound – or alternatively facilitate – long-term therapeutic efficacy. Again, the significance of Lf may be tissue context dependent.

## EFFECTS OF PHAGOCYTES INTERACTING WITH APOPTOTIC TUMOR CELLS

In addition to the release of signaling factors, interaction of apoptotic cells with phagocytes also provides opportunities for regulating tumor cell growth. TAM are the most important phagocytes of apoptotic tumor cells in most cancers, and often prominently display engulfed remnants of apoptotic cells (2, 37, 38). Current work in our laboratory indicates that the TAM of aggressive B-cell lymphoma show up-regulated expression of receptors involved in the recognition and engulfment of apoptotic cells. Furthermore, recent studies in mice have shown that radiotherapy, one of the most important anti-cancer treatment strategies, can enhance tumor cell repopulation *in vivo*, through the induction of apoptosis (4). Such effects may be mediated via responses of macrophages that accumulate as a result of the massive radiation-induced apoptosis as previously proposed (39). Apart from preventing the build-up of free apoptotic cells, removal of apoptotic cells by phagocytosis may therefore drive the pro-tumor activation status of TAM.

Engulfment of apoptotic cells by macrophages has been found to activate downstream signaling pathways that cause the up-regulation and secretion of anti-inflammatory mediators such as IL10, and TGFβ, and the down-regulation of pro-inflammatory mediators such as IL6, IL8, IL12, and TNFα (40–43). Furthermore, incubation of phagocytes with apoptotic cells reduces the effects of LPS, increasing release of IL10, while reducing TNFα, IL1β, and IL12 release. Blocking apoptotic cell engulfment can prevent these responses (44). As well as enhancing anti-inflammatory effects, apoptotic cells have also been shown to promote tumor

growth and angiogenesis. Phagocytes can release growth factors upon engulfment of apoptotic cells, including VEGF (45), and apoptotic cells can induce angiogenesis via electrostatic effects (46).

Given the abilities of apoptotic cells to induce anti-inflammatory signaling, angiogenesis, and the release of growth factors by TAM, it will be important to determine to what extent they influence additional pro-tumor macrophage properties such as matrix remodeling, invasion, and metastasis.

## CONCLUSION

We propose that the apoptotic cell contributes markedly to the conditioning of the tumor microenvironment. Here, we suggest that Lf released from apoptotic cells could contribute to the anti-inflammatory state of the tumor microenvironment. Furthermore, engagement of apoptotic cells by macrophages may also inhibit anti-tumor inflammatory and immune responses, as well as promote tumor cell growth, angiogenesis, and tissue remodeling. These normal, physiological effects of apoptosis endow this fundamental cell death process with regulated and homeostatic properties that permit tissue turnover, organogenesis, and wound healing. However, these properties may be hijacked in malignant disease in order to facilitate cancer progression.

Understanding the complexity of the signaling of apoptotic tumor cells to viable tumor cells, macrophages, and other elements of the tumor environment will be key to improving tumor treatment outcomes and to prevent metastasis, by targeting the interaction of the host with apoptotic cancer cells. This is especially important since most anti-cancer therapies are designed to induce apoptosis of malignant cells, which, without inhibition of these interactions, could ultimately facilitate tumor repopulation.

## ACKNOWLEDGMENTS

Work in the authors' laboratory is supported by Leukaemia and Lymphoma Research, the Medical Research Council (UK), and the Biotechnology and Biological Sciences Research Council (UK).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 08 May 2014; accepted: 13 June 2014; published online: 04 July 2014.*
*Citation: Willems JJLP, Arnold BP and Gregory CD (2014) Sinister self-sacrifice: the contribution of apoptosis to malignancy. Front. Immunol. **5**:299. doi: 10.3389/fimmu.2014.00299*
*This article was submitted to Inflammation, a section of the journal Frontiers in Immunology.*