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The many ways tissue phagocytes respond to dying cells

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Summary

Apoptosis is an important component of normal tissue physiology, and the prompt removal of apoptotic cells is equally essential to avoid the undesirable consequences of their accumulation and disintegration. Professional phagocytes are highly specialized for engulfing apoptotic cells. The recent ability to track cells that have undergone apoptosis *in situ* has revealed a division of labor among the tissue resident phagocytes that sample them. Macrophages are uniquely programmed to process internalized apoptotic cell-derived fatty acids, cholesterol and nucleotides, as a reflection of their dominant role in clearing the bulk of apoptotic cells. Dendritic cells carry apoptotic cells to lymph nodes where they signal the emergence and expansion of highly suppressive regulatory CD4 T cells. A broad suppression of inflammation is executed through distinct phagocyte-specific mechanisms. A clever induction of negative regulatory nodes is notable in dendritic cells serving to simultaneously shut down multiple pathways of inflammation. Several of the genes and pathways modulated in phagocytes in response to apoptotic cells have been linked to chronic inflammatory and autoimmune diseases such as atherosclerosis, inflammatory bowel disease and systemic lupus erythematosus. Our collective understanding of old and new phagocyte functions after apoptotic cell phagocytosis demonstrates the enormity of ways to mediate immune suppression and enforce tissue homeostasis.

Keywords

Dendritic Cells; Monocytes/Macrophages; Autoimmunity; Toll-like Receptors/Pattern Recognition Receptors; Apoptosis/Autophagy; Phagocytosis

Introduction

Cell death is maintained at an equilibrium with cell division in healthy tissues, and is essential for maintaining constant tissue size and proper tissue function. The preferred mode of death under homeostatic conditions is apoptosis, being that this process is conducted neatly where cells shrink and disassemble their contents without damaging neighboring cells. Apoptotic cells express several signals that initiate their engulfment by tissue resident phagocytes. First, a cell undergoing apoptosis will release ‘find-me’ signals that are directly or indirectly produced through activity of the apoptosis executioner caspases, caspase-3 and

caspase-7. They include the partial hydrolysis product of plasma membrane phosphatidylcholine, lysophosphatidylcholine, sphingosine-1-phosphate also known as lysosphingolipid, nucleoside triphosphates, as well as chemotactic factors such as fractalkine/CX3CL1 packaged into microparticles that are released upon apoptosis (reviewed in (1)). Once phagocytes are called in, the second step is identification of the apoptotic cell through 'eat-me' signals including exposure of phosphatidyl-serine (PS) at the outer leaflet of the apoptotic cell plasma membrane *via* the combined activities of plasma membrane flippases and scramblases (reviewed in (2)). In the third step, receptors that mediate apoptotic cell engulfment either bind directly to the exposed PS on apoptotic cells (Stabilin-2 (Stab2), Brain angiogenesis inhibitor (BAI1) and T cell immunoglobulin domain-containing 4 (TIM4)) or indirectly through bridge molecules (Milk fat globule epidermal growth factor (MFG-E8), Growth arrest-specific gene 6 (Gas6), protein S or complement component C1q. The TAM family of receptor tyrosine kinases, TYRO3, Axl and Mer, are an example of the latter receptors (3–6), whereby Mer can bind to either Gas6 or protein S and Axl is constitutively bound to Gas6 in tissue (7). Yet other receptors bind to apoptotic cells independently of PS and these include the phagocytic receptor CD36 which links macrophages to apoptotic cells *via* Thrombospondin-1 acting as a bridge molecule (8). Another example is the LDL-related receptor protein 1 (LRP1 or CD91), which recognizes Calreticulin present on the surface of apoptotic cells or C1q that opsonizes apoptotic cells (9–11). Similarly, the scavenger receptor SCARF1 (12) and the Immunoglobulin superfamily member leukocyte-associated Ig-like receptor 1 (LAIR1, CD305) (13) can both recognize C1q on opsonized apoptotic cells and mediate their phagocytosis.

Successful clearance of apoptotic cells is critical for the preservation of immune tolerance and prevention of autoimmune and chronic inflammatory diseases (14). The prototypical autoimmune disease where a definitive link to apoptotic cell clearance has been made is systemic lupus erythematosus (SLE) (15, 16). Failed clearance of apoptotic cells contributes to their accumulation leading to their death by secondary necrosis which releases inflammatory molecules that activate the innate immune system. Persisting apoptotic cells are the source of self-antigens that can now be presented to the immune system within the context of inflammation, and stimulate the activation of self-reactive T and B cells. Frequently found in SLE patients are anti-double-stranded (ds)DNA, anti-histone, anti-Smith, anti-SS-B/La, anti-ribosomes (Ro) and anti-ribonucleoprotein (RNP) autoantibodies (17, 18). Immune complexes comprised of autoantibody and autoantigen bind to Toll-like receptor (TLR) 7 and TLR9 (19) and trigger the production of interferon (IFN) α , a hallmark of SLE (reviewed in (14, 20)).

Tissue positioning of phagocytes at sites of frequent apoptosis

The orchestrated process of apoptotic cell clearance enables macrophages to locate apoptotic cells within tissues, accurately identify apoptotic cells from viable cells, and initiate their prompt engulfment without interrupting tissue function. For example, in the brain where long-range identification of apoptotic neurons is paramount such as not to disrupt neuronal synapses, the fractalkine receptor CX3CR1 is highly expressed on microglia, the resident macrophages in the brain, and these cells also express high levels of MFG-E8, BAI1, TIM4 and TAM receptors (2, 21, 22). Microglia are abundant within the dentate gyrus, the site of

neurogenesis where large numbers of neural progenitors undergo apoptosis during differentiation into neurons (23–25). Such strategic positioning of macrophages in locations of frequent apoptosis is notable in all tissues, and the location reflects developmental patterns and physiology distinct to each tissue (reviewed in (26)). In the testis, macrophages are located within the interstitium and along seminiferous tubules, but their numbers are highest where undifferentiated spermatogonia concentrate (27). Spermatogenesis is associated with apoptosis of a large proportion of spermatogenic cells (28), and while prompt phagocytosis of these cells can conceivably be conducted by testicular macrophages, this task has so far been assigned to specialized Sertoli cells. These somatic lineage cells nurse spermatogenesis and are located within the seminiferous epithelium alongside developing germ cells (28). Sertoli cells express all three TAM receptors whose signaling is crucial for clearance of apoptotic germ cells during spermatogenesis (5). Sertoli cells also rely on a signaling pathway downstream of BAI involving the cytoplasmic protein Elmo1, which functions with DOCK1 as a guanine nucleotide exchange factor for the GTPase Rac1 to promote actin cytoskeletal rearrangement and apoptotic cell phagocytosis (29). Testicular macrophages, on the other hand, express components of the colony-stimulating factor 1 and retinoic acid pathways to support spermatogonial differentiation (27). ‘Tingible body’ macrophages, a familiar term referring to macrophages containing apoptotic cells or apoptotic cell fragments, are most notable in germinal centers within secondary lymphoid organs after the peak of an immune response when almost 10% of plasma cells undergo apoptosis (30). Notably, the number of ‘tingible body’ macrophages is reduced in the germinal centers of several patients with SLE (31) reflecting the impaired clearance of apoptotic cells in this disease.

Despite the large numbers of cells that undergo apoptosis in tissues on a daily basis, especially in tissues such as the intestine where these numbers in humans approximate 10^{10} daily (32), visualization of apoptotic cell engulfment by phagocytes *in situ* has been difficult due to the prompt and efficient nature of the process. The heterogeneity of professional phagocytes such as macrophages and dendritic cells in tissues adds complexity to the problem. It has presented the questions of which phagocytes in tissues clear dying cells and whether distinct phagocyte subsets are specialized in apoptotic cell uptake *in vivo*. Several studies have resorted to the injection of fluorescently-labeled apoptotic cells into mice to follow their phagocytosis and its immune consequences. Intravenously injected apoptotic B cells or tumor cells were internalized by $CD8\alpha^+$ DC within the spleen and apoptotic cell antigen was cross-presented to antigen-specific CD8 T cells leading to their deletion as a mechanism of immune tolerance (33, 34). Within the pancreatic lymph nodes, $CD11c^+CD11b^+CD8\alpha^-$ DC were found carrying fluorescently-labeled dead cells that had been injected directly into the pancreas (35). Intra-pancreatic injection of dead β -islet cells promoted activation of β cell-reactive T cells, and this was also the case upon the induction of β cell death *in vivo* via injection of Streptozotocin, a cytotoxic chemical that β cells are sensitive to (35). Sub-capsular sinus macrophages lining the lymphatic endothelium overlying B cell follicles were found to capture subcutaneously injected apoptotic cells in an experimental setting (36). Within the lung parenchyma where dendritic cell (DC) subsets are in intimate contact with the bronchial and alveolar epithelium (37, 38), only the DC subset characterized by expression of the E-cadherin-binding integrin αE (CD103) captured intra-

nasally delivered apoptotic thymocytes and trafficked these apoptotic cells to the draining lymph nodes for cross-presentation of apoptotic cell-derived antigens to CD8 T cells (36). The nature of phagocyte that recognizes, samples and/or internalizes apoptotic cells is likely dependent on the tissue and its physiological state at any given time.

Visualizing clearance of cells undergoing apoptosis *in situ*

Rapid turnover of intestinal epithelial cells (IEC) every 4–5 days marks the intestinal epithelium as a site of increased apoptosis during homeostatic conditions (32), and identifies it as an ideal setting for studying apoptotic cell clearance *in vivo*. Using a mouse model where expression of a transgene encoding an enhanced green fluorescent protein (eGFP) and diphtheria toxin receptor (DTR) fusion protein is directed specifically to IECs, the fate of IEC after apoptosis *in situ* could be visualized (39). The model is referred to as the VDTR mouse (39). The diphtheria toxin receptor enables the experimental induction of apoptosis as was determined by administering a carefully titrated concentration of diphtheria toxin that increased the levels of apoptosis over background (39). This leads to the sensitive capture of a larger number of phagocytes in the act of apoptotic IEC uptake, and importantly without causing inflammation or disrupting homeostatic conditions (39). The transgenic VDTR mouse model enabled visualization of phagocytes that had engulfed cells undergoing apoptosis *in situ*. The studies showed that within a heterogeneous network of intestinal lamina propria phagocytes (40, 41), both subsets of macrophages and a single CD103⁺ subset out of three DC subsets are responsible for apoptotic IEC sampling (39). These phagocytes are appropriately positioned for the task; facing the basolateral surface of IEC and capable of extending their dendrites through epithelial tight junctions to sample luminal contents, commensal bacteria (41, 42), and apoptotic IEC (39). After the administration of diphtheria toxin to VDTR mice, both macrophage subsets within the small intestinal lamina propria, identified as CD103⁺CD11b⁺ or CD103[−]CD11b⁺, sampled the bulk of apoptotic IEC over time (39). Because the content of eGFP⁺ cargo identifies phagocytes that had sampled apoptotic IEC, the cells could be subjected to fluorescent activated cell sorting and their transcriptional profiles could then be compared to their eGFP devoid counterparts. A snapshot could be taken over time after the induction of apoptosis to determine the transcriptional changes in phagocytes upon apoptotic cell sampling (39). Analysis of the phagocyte transcriptomes revealed specialized responses, which will be considered next within the larger context of functions phagocytes are known to conduct in response to apoptotic cell sampling.

The first finding from studying the gene expression profiles of phagocytes bearing apoptotic cells was the distinct patterns of expression of apoptotic cell engulfment receptors by macrophages *versus* the CD103⁺ DC (39). The CD103⁺ DC upregulated the gene encoding CD205 which has been reported to detect apoptotic cell ligands (43), while macrophages upregulated genes involved in apoptotic cell clearance including genes encoding the scavenger receptor CD163, the bridge molecule Gas6 and the TAM receptor Mer (39). Other genes were also differentially expressed in macrophages *versus* CD103⁺ DC including upregulation of the genes encoding the fractalkine receptor CX3CR1, TIM4, Stabilin-1, and LRP1 in both macrophages subsets, Axl by CD103[−]CD11b⁺ macrophages, and BAI1, Tyro3 and Stabilin-2 by CD103⁺ DC. Differences in the expression of Axl and Mer have been noted

in murine spleen and liver tissues as well as bone marrow derived macrophages (BMDM) and dendritic cells (BMDC). Axl expression is more abundant than Mer in BMDC and CD11c⁺ splenic DC, and *vice versa*, Mer is more abundant than Axl in BMDM and spleen CD68⁺ 'tingible body' macrophages (7). Tyro3 expression has been noted in BMDC and not BMDM (7). The reasons for these differential patterns of phagocytic receptor expression are presently unclear, but likely serve specialized phagocyte-specific functions in different anatomical sites and conditions.

Defective expression of engulfment receptors has been reported in patients with SLE (reviewed in (15, 44)). Deficiencies in the early components of complement, C1q and C4, are strongly associated with the development of SLE (reviewed in (45, 46)). This association is thought to reflect an impairment in opsonic C1q-mediated phagocytosis of apoptotic cells as well as C1q dependent induction of opsonins such as Gas6 and Protein S (44, 47). Low levels of protein S have been reported in a subset of SLE patients (48). A reduction in the expression of LAIR1 on B cells (49) and plasmacytoid DC (50) from patients with SLE has been reported. Some SLE patients were found to have high serum levels of MFG-E8 at concentrations that *in vitro* inhibit the phagocytosis of apoptotic cells, and may thus lead to similar effects in patients (51). High levels of circulating soluble MER have also been reported in SLE patients (52). When shed upon proteolytic cleavage by the metalloproteinase ADAM-17, the ectodomains of Axl and Mer serve as soluble decoy receptors that interfere with apoptotic cell engulfment (53, 54).

Suppression of inflammation and immune response by apoptotic cells

The well-appreciated immunosuppressive effects of apoptotic cells

One of the best-known consequences of apoptotic cell clearance is immunosuppression. The first evidence came decades ago from observations showing the failure of macrophages to release the pro-inflammatory mediator Thromboxane A2 upon ingestion of apoptotic neutrophils (55), and the ability of apoptotic cells to suppress monocyte production of pro-inflammatory cytokines and induce expression of the anti-inflammatory cytokine IL-10 in response to lipopolysaccharide (LPS) (56). Other studies at the time reported the resolution of acute inflammation by apoptotic cells *in vivo* (57, 58), and this was attributed to the increased levels of the immunosuppressive cytokine TGF- β (59, 60). Apoptotic cells themselves have been reported to produce IL-10 and TGF- β during the process of dying (61, 62). Several studies have demonstrated the induction of immune tolerance in different settings in response to DC phagocytosis of apoptotic cells (reviewed in (63, 64)). The therapeutic potential of apoptotic cell induced immunosuppression has been explored based on data in animal models demonstrating effective control of chronic inflammation in the settings of allograft rejection and graft-*versus*-host disease (65, 66), diabetes (67), arthritis (68–71), septic shock (72), and other chronic and acute inflammatory conditions (reviewed in (73)). A recently completed phase I/IIa clinical trial concluded that a single infusion of early apoptotic donor cells to 13 patients with hematological malignancies a day prior to hematopoietic stem cell transplantation, is a safe and potentially effective prophylactic therapy for acute graft-*versus*-host disease (74). Other studies seeking to promote resolution in neutrophil-dominated inflammation in experimental systems, have devised strategies to

induce neutrophil apoptosis *in situ* via pharmacological inhibition of cyclin-dependent kinases (75), flavones (76), or the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (77). These studies have noted promising results. It is important to note here that the induction of cell death is an important component of drug development efforts, but not all forms of cell death lead to immunosuppression. Achieving the desired anti-inflammatory effects of apoptotic cells is linked to their prompt and efficient clearance by professional phagocytes in order to prevent these cells from undergoing secondary necrosis, which is inflammatory (64). In other instances, such as cancer therapy and some infectious diseases, immunogenic cell death is desirable for mobilizing a protective anti-tumor immune response (reviewed in (78)). The nature of the immune response to dying cells, whether it is tolerance or immunity, is dependent on the signals expressed or released by these cells and the corresponding innate receptor signaling pathways engaged within the responding phagocytes (64).

The receptor systems mediating apoptotic cell-induced immunosuppression

At the forefront of receptors that temper inflammation in response to apoptotic cells are the macrophage and DC expressed TAM receptors, Axl, Tyro and Mer, which are not coincidentally also involved in mediating the phagocytosis of apoptotic cells (79, 80). In a negative feedback loop, the induction of Axl expression by type I IFNs downstream of TLR engagement leads to Axl binding to the type I IFN receptor, subsequent upregulation of the suppressor of cytokine signaling proteins SOCS1 and SOCS3, and termination of the inflammatory response (81). The expression of TAM receptors can also be induced by apoptotic cell recognition, as seen in intestinal lamina propria macrophages and CD103⁺ DC (39), presumably to prevent the initiation of immune responses. Mice deficient in all three members, Tyro, Axl, and Mer, develop chronic inflammation, enlarged secondary lymphoid organs, autoantibodies and systemic autoimmunity (82). The pathological phenotype observed is likely a combination of impaired apoptotic cell clearance and hyper-activation of B and T lymphocytes in secondary lymphoid organs.

Another set of receptors that have been associated with the immunosuppressive effects of apoptotic cells are the nuclear hormone receptors peroxisome-proliferator-activated receptor (PPAR) and liver x receptor (LXR). These receptors are additionally important for apoptotic cell clearance (83–86). The first report implicating PPAR γ in apoptotic cell-induced immunosuppression demonstrated its activation in response to apoptotic cell uptake, and its attenuation of reactive oxygen species production by inflammatory peritoneal macrophages (83). The homeostatic clearance of apoptotic neutrophils has been linked to LXR such that neutrophils accumulate within the blood and tissues of LXR-deficient mice (87, 88). Interestingly, LXR activation upon apoptotic neutrophil engulfment represses transcription of the inflammatory cytokine IL-23 (88), and in this manner controls the levels of neutrophils in the blood by targeting the upstream regulator of IL-17 and granulocyte-colony stimulating factor (G-CSF) that are important for granulopoiesis (89). Global or macrophage-specific deletion of PPAR δ or deficiency in LXR α/β or retinoid X receptor (RXR) α , which heterodimerizes with LXR, leads to defects in apoptotic cell clearance *in vivo* and consequent increases in serum levels of autoantibodies with progressive lupus-like autoimmune disease (84, 86, 90). The phenotype of these mice is similar to that of mice with

impaired apoptotic cell removal (91). Apoptotic cell uptake by PPAR δ , RXR α or LXR α / β deficient macrophages is unable to suppress LPS-induced IL-12, TNF- α , or IL-1 β transcription and synthesis, and fails to induce TGF- β and IL-10 synthesis demonstrating that PPAR δ , RXR α and LXR contribute to the anti-inflammatory response induced by apoptotic cells (84, 86, 90).

Lastly, Nr4a1, a member of the Nr4a subfamily of nuclear receptors is induced in resident peritoneal macrophages in response to the phagocytosis of apoptotic cells in a PS-dependent manner (92). Nr4a1 expression interferes with NF- κ B signaling to repress the expression of inflammatory cytokines such as IL-12 and maintain self-tolerance in the face of increased cell death in a model of experimental murine lupus (92).

Subset-specific pathways of suppressing inflammation

The studies tracking apoptotic IEC into small intestinal lamina propria phagocytes have shown that phagocytes adopt two prominent transcriptional signatures in response to apoptotic cells: the ‘downregulation of inflammatory genes’ and ‘suppression of the immune response’ (39). No upregulated genes were shared among these phagocytes, and only two genes were down-modulated by all three subsets (39): *Plac8* implicated in clearance of certain microorganisms (93), and *Itgb7* encoding integrin- β 7 that mediates homing to gut-associated lymphoid tissue. Integrin- β 7 is notably the target for a current IBD therapy with humanized monoclonal antibodies Vedolizumab and Etrolizumab (94). Different sets of genes are downregulated in DC *versus* macrophages, some of which have not previously been associated with the response to apoptotic cells. Genes encoding pattern recognition receptors *Clec4a*, *Clec4b1*, *Cd209a*, and *Tlr2* are downregulated in CD103⁺CD11b⁺ and CD103⁻CD11b⁺ macrophages (39). TLR2 mediates the innate response to fungal β -glucans and bacterial peptidoglycan, lipoproteins and lipoteichoic acid (95). *Clec4a* encodes DCIR, a C-type lectin receptor and member of the dendritic cell immunoreceptor (DCIR) family that was first described as an attachment factor for human immunodeficiency virus (HIV)-1 in DC (96). DCIR has also been reported to potentiate DC cross-presentation of antigen to CD8 T cells (97) and sustain type I IFN signaling (98). Notably, a polymorphism in DCIR, SNP rs2377422, is a genetic susceptibility factor for SLE and Sjogren’s syndrome (99), two autoimmune diseases where excessive apoptosis and impaired apoptotic cell clearance contribute to disease pathogenesis (15, 100). *Clec4b1* encodes DCAR, a different member of the DCIR family that delivers antigens to the endocytic pathway in DC and enhances antigen-specific T cell responses (101). *Cd209a* encodes the C-type lectin CD209a, the murine homolog of human DC-specific ICAM-3-grabbing non-integrin DC-SIGN or CD209, which has been reported to induce the development of pathogenic T helper 17 cells driving severe hepatic granulomatous inflammation in a murine model of schistosomiasis (102). Intestinal lamina propria CD103⁺CD11b⁺ and CD103⁻CD11b⁺ macrophages also downregulated *Alox5ap* encoding arachidonate 5-lipoxygenase (39), which facilitates inflammatory leukotriene biosynthesis (103). 5-lipoxygenase associates to lipid rafts of apoptotic cell membranes and presumably leads to the synthesis of apoptotic cell ligands that stimulate PPAR γ in macrophages (85).

On the other hand, intestinal lamina propria CD103⁺ downregulated transcripts related to components of the inflammasome including *Nlrp3*, *Casp1*, and *Il1a* (104), as well as genes encoding MAPK/ERK signaling proteins including *Lrrk2*, *Map3k4*, and *Fos*. Leucine rich repeat kinase 2 (LRRK2), is a multifunctional protein kinase known to be a prominent contributor to familial Parkinson's disease (105). Genome wide association studies have also identified LRRK2 as a major susceptibility gene for Crohn's disease (106–108). Notably, the Parkinson's disease linked LRRK2(R1441G) mutation has been associated with increasing pro-inflammatory cytokine secretion (109, 110), and certain LRRK2 polymorphisms may function similarly in IBD. Other genes that overlapped with IBD susceptibility loci and were modulated upon apoptotic IEC sampling included *Pfkfb3* encoding PFKFB3 (the inducible isoform of 6-phosphofructo-2-kinase, iPFK2) which was upregulated in apoptotic IEC bearing CD103⁺ DC (39). PFKFB3 is a PPAR γ target (111, 112) involved in active PPAR γ -dependent suppression of diet-induced intestinal inflammation (113). Curiously, *IL12b* encoding the p40 subunit shared by IL-12 and IL-23, in which single nucleotide polymorphisms are associated with Crohn's disease in Korean populations (114), was upregulated in apoptotic IEC bearing intestinal lamina propria CD103⁺ DC (39).

Classical and alternative complement genes *C1qa* (encoding complement component C1qa) and *Cfp* (encoding complement factor Properdin), respectively (115), were downregulated in CD103⁺ DC (39). While this may be counterintuitive in light of the role that complement initiators and Properdin play in the recognition and opsonic phagocytosis of apoptotic cells (116), dampening activation of the complement cascade may supersede these functions while also attenuating NLRP3 inflammasome activation (117, 118). Additionally, a regulator of complement activation *Cfh* encoding complement factor H that attenuates late steps of complement activation, is upregulated in CD103⁺CD11b⁺ macrophages (39). Factor H binds to apoptotic cells and when internalized catalyzes the generation of intracellular C3b, which opsonizes the apoptotic cell surface to promote clearance and aid in preventing autoimmunity (reviewed in (116)).

A DC-specific upregulation of negative immune response regulators

Of the phagocytes sampling apoptotic IEC, CD103⁺ DC upregulated genes involved in the negative regulation of inflammatory signaling (39). Notably, CD103⁺ DC appeared to be the only cells to do so. A common feature of the negative regulators is that they target more than one pathway of inflammation including the inflammasome, type I IFN, and MAPK signaling. This makes them ideal for enforcing immunosuppression at more than one level and in the face of more than one type of inflammatory stimulus. An example of a gene whose expression is induced in response to apoptotic cells is *Nlr5* (39), which encodes a member of the Nod-like receptor family NLRC5 and works to inhibit both NF- κ B and type I IFN signaling (119). NLRC5 suppresses the activation of IKK by TLR ligands through blocking the assembly of a functional IKK complex by interacting directly with the IKK catalytic subunits, IKK α and IKK β , to inhibit their phosphorylation and block their association with NEMO (119). NLRC5 also binds to the RIG-I like receptors, RIG-I and MDA-5, which are activated by double-stranded and single-stranded DNAs or certain viruses (120), and prevents their association with the mitochondrial anti-viral signaling protein MAVS and induction of a downstream type I IFN response (119).

Another example of a negative regulator that targets more than one pathway of inflammation is the protein A20 encoded by *Tnfrsf3*, and whose expression is upregulated in small intestinal lamina propria CD103⁺ DC in response to apoptotic IEC (39). Mice deficient in A20 exhibit severe systemic inflammation, cachexia, increased sensitivity to LPS and TNF, and premature death (121). Deletion of A20 in specific cell types in mice leads to autoimmunity and organ-specific inflammation (122–128) similar to those in humans identified to have polymorphisms and mutations in *Tnfrsf3* gene (129, 130). Deletion of A20 in DC leads to the development of pathologies in mice similar to those seen in humans with inflammatory bowel disease and SLE including colitis, ankylosing spondylitis, autoantibody development, nephritis, and splenomegaly (123, 125). A20 is a ubiquitin modifying enzyme that potently inhibits NF- κ B signaling (131, 132), RIG-I signaling and antiviral responses (133, 134), as well as NLRP3 inflammasome activation (135, 136). A20 inhibits *Nlrp3* transcription as a consequence of its inhibition of NF- κ B signaling (135), but also exerts distinct effects on the function of the NLRP3 inflammasome complex by regulating its ubiquitination and composition (136). Enhanced apoptotic cell phagocytosis, presentation of apoptotic cell-derived antigen, and activation of self-reactive T cells has also been associated with A20 deficiency in DC (125). Notably, apoptotic cell uptake by A20 deficient DC fails to inhibit pro-inflammatory cytokine production in response to LPS (125). DC induction of A20 in response to apoptotic cells (39) may thus serve not only to inhibit inflammation, but also apoptotic cell antigen presentation. Combined with a shut down in apoptotic cell phagocytosis in a negative feedback loop (125), A20 might serve to limit the supply of self-antigen and its presentation within an inflammatory context.

Finally, CD103⁺ DC bearing apoptotic IEC show higher expression of *Oas1* transcripts compared to their devoid counterparts (39). Unlike the interferon-induced 2'-5'-oligoadenylate synthetases, the *Oas1* encoded interferon inducible oligoadenylate synthetase-like 1 (OASL1) is a non-enzymatic OAS protein that has two ubiquitin-like repeats (137). OASL1 inhibits the translation of IRF7, the master transcription factor for type I IFN, by binding to the 5' untranslated region of the *Irf7* mRNA (138). Apoptotic cell-carrying CD103⁺ DC also express higher levels of *Spred1* (39), encoding for Spred1, which is highly expressed in hematopoietic cells (139) and belongs to the Sprouty (Spry) family of highly conserved proteins that modulate growth factor mediated mitogen-activated protein kinase activation (140, 141). The negative regulatory effects of Spred-1 have been shown in the context of allergen-induced airway eosinophilia and hyper-responsiveness as well as IL-3 signaling in hematopoietic cells (139, 142). Collectively, the transcriptional expression patterns discussed in this and the previous section reflect an overall genome-wide transcriptional suppression of inflammatory genes in response to apoptotic IEC sampling *in situ*.

Metabolic homeostasis in phagocytes post an apoptotic cell meal

When macrophages internalize apoptotic cells, they acquire the lipids, carbohydrates, protein, and nucleotides from the apoptotic cell. This content increases depending on the number of apoptotic cells a macrophage ingests, and poses a significant metabolic burden on the macrophage (143). Several studies have now shed light on the metabolic changes macrophages undergo to restore normal cellular function and homeostasis.

Increased lipid metabolism

Cholesterol derived from an engulfed apoptotic cell becomes incorporated into the cholesterol pool of engulfing macrophages increasing their cholesterol content by several fold (144). Several pieces of evidence show the mobilization of a homeostatic response aimed at restoring macrophage lipid metabolism. The increased levels of cholesterol in phagocytes following apoptotic cell phagocytosis had originally prompted a role for the fatty acid and sterol sensing nuclear receptors, PPAR and LXR, in apoptotic cell clearance. Apoptotic cell engulfment was found to induce the activation and expression of PPAR γ (83, 145), PPAR δ (90), and LXR (86) in macrophages. PPARs and LXRs form heterodimers with their obligate partners, the RXRs following binding to fatty acids and oxysterols, and recruit coactivators to induce transcription of a variety of genes involved in lipid and cholesterol metabolism (146, 147). Within the small intestinal lamina propria, increased expression of genes involved in lipid processing is notable in lamina propria resident macrophages that had engulfed apoptotic IEC (39). Intestinal CD103⁺CD11b⁺ macrophages upregulate the expression of *Abca1*, a direct target of LXR that encodes an ATP-binding cassette transporter ABCA1 also known as the cholesterol efflux regulatory protein (39). J774 macrophages and primary macrophages in tissue culture had similarly been reported to increase *Abca1* transcription after the addition of apoptotic cells (144, 148). ABCA1 mediates the transport of cellular free cholesterol and phospholipid across the plasma membrane and transfers them primarily to circulating apolipoprotein A-I, leading to the formation of high density lipoprotein (HDL) particles (149). In turn, a small fraction of HDL, called Pre β -1 HDL, interacts with ABCA1 to remove cholesterol and phospholipids from cells (150). The exposure of PS on apoptotic cells is necessary and sufficient for both the upregulation of ABCA1 expression and the efflux of cholesterol (144). PS-dependent cholesterol efflux also requires the activity of PPAR γ and LXR, suggesting communication between these nuclear receptors and PS-recognizing engulfment receptors (144). Evidence to date shows that signaling through the PS-specific phagocytic receptor BAI1 contributes to ABCA1 upregulation, albeit this was independent of LXR (148).

In macrophages, PPAR γ controls the expression of CD36, transglutaminase-2, pentraxin-3, and Axl, all of which facilitate apoptotic cell phagocytosis (145, 151). PPAR δ controls the expression of bridge molecules such as C1qb, Gas6, and Thrombospondin-1, and its deletion impairs the phagocytosis of apoptotic cells (90). Similarly, apoptotic cell clearance was reduced in macrophages deficient for both LXR α and LXR β (86). Heterodimerization of RXR α with PPAR γ or LXR also controls the expression of Mer (84, 86).

Apoptotic cell uptake induces macrophage expression of uncoupling protein 2 (UCP2) (152), a finding that reflects the ability of fatty acids, such as those derived from apoptotic cells, to enhance *Ucp2* transcription through the activation of PPARs (153, 154) and the sterol regulatory element binding protein (SREBP) (154, 155). UCP2 is a mitochondrial anion carrier protein that uncouples oxidative phosphorylation from ATP production by dissipating protons across the inner mitochondrial membrane, and also decreases reactive oxygen species (ROS) produced by electron transport (154). Fatty acids also regulate the activity of UCP2 by increasing its proton conductance across inner mitochondrial membranes (156). By lowering mitochondrial membrane potential, which inversely controls

engulfment capacity, UCP2 enables macrophages to internalize more than one apoptotic cell at a time (152).

An increased level of *AcadSB* transcripts encoding the short/branched chain specific acyl-CoA dehydrogenase (ACADSB, also known as 2-methylbutyryl-CoA dehydrogenase) has been noted in intestinal lamina propria CD103⁺CD11b⁺ macrophages containing apoptotic IEC (39). This mitochondrial matrix enzyme is a member of the acyl-CoA dehydrogenase (ACAD) family of enzymes which catalyze the dehydrogenation of acyl-CoA esters as the first step in the metabolism of fatty acids (157). ACADSB has the greatest activity toward short branched chain acyl-CoA derivatives particularly 2-methylbutyryl-CoA in the isoleucine pathway, isobutyryl-CoA and 2-methylhexanoyl-CoA, as well as short straight chain acyl-CoAs such as isobutyryl-CoA and hexanoyl-CoA. Mutations in *AcadSB* are associated with a rare inherited metabolic disorder, 2-methylbutyrylglycinuria, which manifests in seizures and psychomotor delays from a defect in L-isoleucine catabolism (158–160).

Breakdown of macrophage lipid metabolism – the case of atherosclerosis

The regulation of lipid metabolism in macrophages upon phagocytosis of apoptotic cells extends past the maintenance of cell-autonomous homeostasis and occupies center stage in atherosclerosis. Elevated plasma levels of low density lipoprotein cholesterol (LDL-C) remain a strong risk factor for atherosclerosis (161, 162). The retention of these particles within the intima of arteries along with the production of ROS, lipid peroxidation products and inflammatory mediators, leads to the development of atherosclerotic plaques (163). Here, foam cells – macrophages that have ingested large amounts of cholesteryl fatty acids – accumulate along with necrotic cells arising from the failed clearance of apoptotic macrophages. Over time, the arterial wall breaks down, and the resultant activation of pro-coagulant and pro-thrombotic pathways precipitates occlusive thrombus formation and myocardial infarction (163). Atherosclerosis is the culmination of interrelated events including defective resolution of the chronic inflammation triggered by lipoproteins retained in the subendothelium, defective egress of inflammatory cells and impaired clearance of apoptotic macrophages (163–165).

Notably, several of the genes involved in regulating lipid metabolism in macrophages have been closely associated with atherosclerosis. Genetic deficiency for *ABCA1* reduces the plasma levels of HDL cholesterol (HDL-C), one of the parameters associated with higher risk for cardiovascular disease (149, 150, 166). In mouse models of atherosclerosis, macrophage specific deletion of *ABCA1* resulted in a large accumulation of cholesterol in macrophages, and exacerbated atherosclerosis in the advanced stage of disease (167). *ABCA1* also protects macrophages from undergoing apoptosis after the engulfment of apoptotic cells, and it may do so in two ways. First, the efflux of cholesterol would protect macrophages from endoplasmic reticulum stress-induced apoptosis in response to the accumulated cholesterol (168). In fact, *ABCA1* upregulation in response to apoptotic cell uptake might make macrophages resistant to the extreme conditions of cholesterol loading that they encounter within atherosclerotic lesions (169). Second, the nascent HDL formed through the activity of *ABCA1* protects macrophages from the oxidative burst induced by

the increased content of oxidized phospholipids derived from internalized apoptotic cells (170). Deficient ABCA1 function could thus contribute to increased numbers of apoptotic macrophages and resultant necrosis within atherosclerotic plaques. Consistent with these observations, transgenic overexpression of BAI1, which induces *Abca1* transcription, within a hyperlipidemic genetic background of deficiency for the low-density lipoprotein receptor 1 (LDLR1) improved the HDL/low density lipoprotein (LDL) ratios of mice fed an atherogenic Western diet (148). Conversely, BAI1 deficiency under the same conditions resulted in significantly reduced levels of serum lipids including total cholesterol and HDL (148).

Genetic polymorphisms in UCP2 have been associated with several metabolic diseases including obesity, diabetes, and atherosclerosis (154). UCP2 has been linked to the regulation of the size of atherosclerotic plaques in mouse models (171, 172). This is likely a reflection of the increased levels of local ROS and ROS-driven lipid oxidation as well as the impaired clearance of apoptotic cells. In fact, deficiency in many of the apoptotic cell engulfment receptors or bridging molecule, such as transglutaminase-2, MFG-E8, Thrombospondin-1, complement C1q, Gas6 and pentraxin-3 (all targets for PPARs and LXR, see above) has identified specific roles for these molecules within advanced atherosclerotic lesions in mouse models (173–178). Notably, deficiency in Axl restricted to the hematopoietic compartment of athero-prone mice did not affect plaque inflammation, apoptotic cell clearance or disease progression, and this did not appear to be due to compensatory Mer functions (179). LXR expression in macrophages is critical for the reduction of atherosclerosis and concordantly, ligand activation of LXR and PPAR reverses atherosclerosis in mouse models (180, 181). However, the efficacy of these agonists in preventing cardiovascular disease in humans remains to be seen (182).

Several studies have also elucidated an important function for Mer in atherosclerosis. As mentioned above, Mer is a prominent target of LXR and as such its expression is regulated by increased levels of macrophage fatty acids and cholesteryl esters – as would be the case for foam macrophages in atherosclerotic lesions – and increased Mer expression enhances the efficiency of apoptotic cell clearance. Accordingly, Mer deficiency in mice prone to atherogenesis increases the numbers of uncleared apoptotic cells and necrotic areas within atherosclerotic plaques (183, 184). Impaired Mer function upon proteolytic cleavage of its extracellular domain by metalloproteinase ADAM-17 generates soluble Mer, which competes with Gas6 for binding to Mer, and disables its functions in apoptotic cell clearance and suppression of inflammation (54, 185–187). Expression of a cleavage resistant form of Mer in the hematopoietic compartment of atherogenesis-prone LDLR1 deficient mice fed an atherogenic high-fat diet, enhanced apoptotic cell clearance, decreased plaque necrosis, and increased resolution (188). Analysis of carotid endarterectomy samples revealed a strong positive correlation between soluble MER levels and both necrosis within lesions and the manifestation of an ischemic attack or stroke (188). CD68⁺ macrophages nearest to the necrotic cores of human carotid plaques exhibit highest ADAM17 and lowest surface MER expression (189).

The induction of regulatory CD4 T cells - a specialty of DC carrying apoptotic cells

Apoptotic cell-induced TGF- β and T_{REG} cells

One of the major ways whereby innate recognition of apoptotic cells induces immune tolerance and suppresses inflammation is by creating the ideal conditions for the development of immunosuppressive regulatory CD4 T (T_{REG}) cells. These cells are marked by expression of the transcription factor Foxp3 and function to control organ-specific autoimmune diseases by suppressing the activation of autoreactive T cells, presumably to self-antigens such as those derived from apoptotic cells (190, 191). Phagocytosis of apoptotic cells *in vitro* leads to the production of TGF- β , IL-10 and the vitamin A metabolite retinoic acid (60, 192–198). These molecules provide the cytokine milieu for the differentiation of induced T_{REG} cells (199–205), which arise in the periphery and comprise a population distinct from natural T_{REG} cells that develop in the thymus (206). One of the conserved non-coding DNA sequences (CNS1) within the Foxp3 promoter in T_{REG} cells is responsive to TGF- β (207), contains Smad3 and retinoic acid receptor binding sites, and is required for differentiation of induced T_{REG} cells specifically in the gut-associated lymphoid tissues and mesenteric lymph nodes (MLN) (208). Intestinal lamina propria and MLN CD103⁺ DC uniquely drive FOXP3⁺ T_{REG} cell generation (203, 205, 209, 210). Intestinal lamina propria CD11b⁺F4/80⁺ macrophages have also been shown to induce FOXP3⁺ T_{REG} cells *in vitro* (211). Colonic CD11c⁺CD11b⁺F4/80⁺ cells, thought to be CX₃CR1⁺ macrophages, maintain FOXP3 expression as well as suppressive activity of T_{REG} cells during colitis *in vivo* through the production of IL-10 (212).

Studies *in vitro* have shown that DC phagocytosis of apoptotic cells instructs naïve CD4 T cell differentiation into T_{REG} cells (192, 198). DC expression of the α v integrin family of apoptotic cell receptors, α v β 3 and α v β 5, enables binding to and phagocytosis of apoptotic cells *via* bridge molecules such as Thrombospondin-1 and MFG-E8 (213). Intestinal CD103⁺ and not CD103⁻ DC could induce T_{REG} cell differentiation without exogenously added TGF- β (213, 214). Intestinal CD103⁺ DC express the α v β 8 integrin, which activates latent TGF- β and induces T_{REG} cell generation (213, 215–217). These studies strongly implicate apoptotic cell-stimulated TGF- β production in the generation of T_{REG} cells. Evidence *in vivo* linking T_{REG} cells to apoptotic cell engulfment comes from several studies. Myeloid specific deletion of the integrin α v or β 8 decreases T_{REG} cell numbers in the intestine (213, 218). The infusion of apoptotic cells into mice expands the numbers of inducible T_{REG} cells (61, 219, 220), and this requires the presence of macrophages (61). Experimental induction of apoptosis within the intestinal epithelium of mice leads to an expansion of induced T_{REG} cells in MLN (39). DC carrying apoptotic IEC have been noted in the MLN (39, 221, 222), suggesting that T_{REG} cells induced by such DC could have specificity to epithelial cell derived self-antigens.

Specialization in T_{REG} cell differentiation is restricted to apoptotic cell carrying DC

A comparison of the transcriptional profile of apoptotic cell containing DC *versus* macrophages within the small intestinal lamina propria reveals a distinct 'regulatory CD4 T cell activation' signature that is confined to CD103⁺ DC and not CD103⁺CD11b⁺ or

CD103⁺CD11b⁺ macrophages (39). These CD103⁺ DC upregulate *Aldh1a2* (39), which catalyzes the conversion of retinal to retinoic acid to generate T_{REG} cells in the intestine (199, 203, 204). Also upregulated is *Lrrc32* (39), which encodes LRRC32 (also known as GARP). Expression of this protein has been studied on T_{REG} cells where it associates with and retains latent TGF- β on the cell surface to promote T_{REG}-mediated suppression (223). The expression of GARP by DC in response to apoptotic cell sampling (39) may also serve a similar role. Among the phagocytes that sample apoptotic IECs, highest expression of CCR7, the chemokine receptor that mediates DC migration to lymph nodes, is found on CD103⁺ DC that had sampled apoptotic cells (39), and these DC also have upregulated *Cd40* (39) which encodes the costimulatory molecule CD40 whose interaction with CD40L regulates T_{REG} cell numbers under homeostatic conditions (224, 225). Also upregulated by CD103⁺ DC in response to apoptotic cell sampling is *RelB* encoding the Rel/NF- κ B transcription factor family member RelB that shows constitutively active κ B-binding activity in lymphoid tissues under steady state conditions (226). Curiously, steady state migratory DC in peripheral lymph nodes characterized by the expression of CD40, nuclear RelB and surface latent TGF- β have been reported to transport peripheral self-antigens to the draining lymph nodes in a CCR7-dependent manner, present them to T cells and induce the differentiation of naïve CD4 T cells into T_{REG} cells (227).

Small intestinal lamina propria CD103⁺ DC that have sampled apoptotic cells upregulate the suppressive co-stimulatory molecule *Cd274* (Programmed death ligand PD-L1) (39), which synergizes with TGF- β to promote the instruction of T_{REG} cell differentiation by DC, and whose expression on T_{REG} cells has been reported to promote their differentiation and maintain their function by enhancing Foxp3 expression (228). Also upregulated by these CD103⁺ DC in response to apoptotic cell sampling are the chemokine encoding genes *Ccl22* and *Ccl17* that have been found to induce efficient chemotaxis of intestinal lamina propria T_{REG} cells (229). Interestingly, CCL22 and CCL17 were reported to be selectively expressed by small intestinal lamina propria DCs at high levels, and that T_{REG} cells were in close proximity to these DCs (229). The collective upregulation of these genes upon apoptotic IEC sampling by lamina propria CD103⁺ DC sets the stage for instructing differentiation of T_{REG} cells (39), enabling DC to directly impact the generation of these critical mediators of intestinal tolerance and homeostasis (203).

Within the MLNs, migratory CD103⁺ DC containing apoptotic cells share a transcriptional profile similar to equivalent apoptotic cell carrying CD103⁺ DC in the small intestinal lamina propria (39). Several transcripts involved in T_{REG} cell differentiation are additionally increased in migratory MLN CD103⁺ DC relative to equivalent MLN DC that do not contain apoptotic cells, including *Ido1* and *Ii10* (39). IL-10 is a critical player in the induction of T_{REG} cells by tolerogenic DC (230, 231). *Ido1* encodes indoleamine 2,3 dioxygenase-1 (IDO-1) whose expression in cultured BMDC is induced by apoptotic cells (232, 233), and whose activity can be induced by TGF- β (234). Furthermore, the administration of apoptotic cells to mice induces the expression of IDO in splenic marginal zone macrophages (235). IDO-1 promotes immune tolerance through various mechanisms including the suppression of effector T cell responses and IL-6 production by DC, as well as the induction of T_{REG} cell differentiation (235–238). IDO-1 also suppresses IL-12 production and induces IL-10 and TGF- β production by macrophages exposed to apoptotic cells (239, 240). This is dependent

on its downstream effector, the metabolic-stress sensing protein kinase GCN2 (240). Myeloid specific deletion of GCN2 in lupus-prone mice increases pathology and mortality while agonist-driven activation of GCN2 protects those mice from disease (235).

Compared to apoptotic cell carrying CD103⁺ DC in the small intestinal lamina propria, MLN CD103⁺ DCs carrying apoptotic IECs express approximately 4-fold lower levels of *Itgb8*, which converts TGF- β into an active state, while the levels of *Tgfb1* transcripts are comparable (39). Migratory apoptotic cell carrying DC may thus already be primed to induce T_{REG} cells upon arrival in the MLN. Within the MLN, T cell co-stimulatory receptors (CD274, CD40), and secreted signals (retinoic acid (*via Aldh1a2*), CCL22), are higher in apoptotic cell carrying CD103⁺ DC relative to CD103⁺ DC that do not contain apoptotic cells (39). Consistent with this profile, the apoptotic cell content of CD103⁺ DC is the determining factor in their ability to induce Foxp3⁺ T_{REG} cells *ex-vivo*, and notably without added TGF- β (39).

Degradation of apoptotic cell DNA

Apoptotic cell chromosomal DNA is fragmented *via* cell autonomous and macrophage dependent mechanisms (241). DNASE1 facilitates the breakdown of chromatin during apoptosis, and DNASE-I deficient mice develop SLE-like syndrome with anti-nuclear antibodies, glomerular immune complex deposition and glomerulonephritis (242). Lower levels of DNASE-1 have been noted in SLE patients correlating with high antibody titers against nucleosomal antigens (242–244). Within cells undergoing apoptosis, the fragmentation of DNA into nucleosomal units is the sole responsibility of the Caspase-activated DNase (CAD or DFF-40), which under non-apoptotic conditions is kept in check by its specific inhibitor (ICAD or DFF-45) (245).

DNASE1L3, a circulating DNase produced primarily by macrophages and DC, digests the genomic DNA in apoptotic cell derived membrane-bound vesicles called microparticles (246). These microparticles are smaller than apoptotic bodies and can be found in the plasma of SLE patients bound to IgG to form a type of immune complex that may contribute to pathogenesis (247, 248). A highly basic C-terminal peptide in DNASE1L3 enables its association with liposomes encapsulating DNA (249), and this property of DNASE1L3 is thought to enable its digestion of surface-exposed chromatin exposed on microparticles thereby preventing autoantibody binding (246, 250). *Dnase1l3*-deficient mice develop antibodies to dsDNA and chromatin and exhibit features of SLE including glomerulonephritis (246).

Although apoptotic cell DNA is rapidly degraded by circulating DNASEs, DNA is bound to autoantibodies in SLE or the cationic antimicrobial peptide LL37 in psoriasis, an autoimmune disease of the skin (251, 252). These complexes protect the DNA from degradation and deliver it *via* endocytosis to endosomal compartments within DC where it engages TLR9 and signals downstream type I IFN and pro-inflammatory cytokine production (251–254). In psoriasis, LL37 can similarly interact with self RNA and trigger TLR7 and TLR8 in human DC (255). DNA and RNA containing immune complexes internalized by autoreactive B cells *via* the B cell receptor also gain access to endosomal

compartments within B cells where they engage TLR9 and TLR7, respectively, and stimulate B cell proliferation (256, 257) (and reviewed in (19, 258)).

After phagocytosis by macrophages, apoptotic cell DNA is degraded within lysosomes by lysosomal DNASE2 (259, 260). Mice deficient in CAD or ICAD do not exhibit features of autoimmunity because apoptotic cell DNA is degraded by lysosomal DNASE2 after macrophage phagocytosis of CAD-deficient apoptotic cells (245). Deletion of *Dnase2* in mice is embryonic lethal due to constitutive production of interferon- β (260). Lethality can be rescued by crossing to a deficiency in type I IFN receptor (261), but as they age those mice develop chronic polyarthritis resembling human rheumatoid arthritis (260). This phenotype is a result of TNF- α production by macrophages unable to degrade apoptotic cell DNA (260). DNASE2 deficiency in both the hematopoietic and stromal compartment is required for the development of arthritis in these mice (262). Deletion of TLR3, TLR9 or both TLR signaling adaptors TRIF and MyD88, did not impact the lethality of DNASE2 deficient mice suggesting that the interferon response is mediated by a TLR-independent mechanism (263). Analysis of DNASE2 deficient mouse fetuses showed liver and spleen accumulation of cyclic dinucleotide [G(2',5')pA(3',5')p] (2',3'-cGAMP), the product of the cytoplasmic DNA sensor cyclic GMP-AMP synthase (cGAS) (264). DNASE2 deficient mouse embryonic fibroblasts produced 2',3'-cGAMP in a cGAS-dependent manner during apoptotic cell engulfment (264). These results suggest that undigested apoptotic cell DNA in lysosomes gains access to the cytoplasm of the engulfing cell to activate cGAS and type I IFN production. Indeed, mice doubly deficient for DNASE2 and the 2',3'-cGAMP-responsive protein stimulator of interferon genes (STING) – downstream of cGAS – were rescued from lethality and polyarthritis even though macrophages from these mice could not digest engulfed apoptotic cell DNA (265). Cytoplasmic DNA sensors rather than TLR9 are involved in DNASE2 deficient mice because in the absence of DNASE2, the DNA fragments necessary to engage TLR9 are no longer generated (266). In cells undergoing apoptosis through the mitochondrial pathway, the activation of caspases 9, 3 and 7 suppresses the production of type I IFN production in response to released mitochondrial DNA, which would otherwise activate the cGAS-STING pathway (267, 268). This evidence collectively points to the cGAS-STING pathway as a major driver of type I IFN in response to undigested apoptotic cell derived mitochondrial and chromosomal DNA. However, it is important to add a distinction here. Autoantibodies to nuclear antigens persist in DNASE2 and STING double knock-out mice, but they are no longer detectable on a background deficient for Unc93b1, the ER-associated chaperone for TLR7 and TLR9 trafficking to endosomes (269, 270). Of note, these autoantibodies were not specific to dsDNA but to RNA-associated antigens perhaps reflecting the excessive accumulation of apoptotic cell debris containing RNA-associated autoantigens (270). Collectively, the data reveal that lingering undigested DNA from apoptotic cells triggers a STING-dependent type I IFN response and TLR-dependent autoantibody production.

Paradoxically, STING has also been identified as a negative regulator in systemic autoimmunity driven by TLR activation (271). The surprising evidence comes from the observation that STING deficiency in autoimmune prone MRL/*lpr* mice leads to the development of more severe disease and shorter lifespan (272). In line with these findings, STING deletion abrogates the hallmark apoptotic cell-induced TGF- β , IL-10 and IDO

production by DC, and these DC produce pro-inflammatory IL-6 instead (273, 274). It will be important to decipher the mechanisms underlying the suppressive activities of STING in the context of autoimmunity and the response to apoptotic cells.

In conclusion – one more aspect of the biology of apoptotic cell clearance in tissues

The breadth of functions that phagocytes orchestrate in response to apoptotic cells is truly remarkable, as illustrated here by the plethora of homeostatic and pathological conditions impacted by each step in the process of recognizing, sampling and internalizing apoptotic cells. The scope is large and cannot be covered in a single review (please see other recent reviews (275–278)). One final point to make pertains to the additional role that non-professional phagocytes, such as epithelial cells, play in the clearance of apoptotic cells. This has been documented in the respiratory, colonic, and post-weaning mammary epithelium (279–283). To add yet another layer of complexity, macrophages also play a role in this setting. During phagocytosis of apoptotic cells or under inflammatory conditions, macrophages release insulin-like growth factor 1 (IGF-1), which binds to IGF-1 receptor (IGF1R) specifically expressed on epithelial cells and decreases their appetite for apoptotic cells (284). Macrophages also release microvesicles and through IGF-1 redirect epithelial cells towards internalizing microvesicles instead (284). The preferential uptake of microvesicles over apoptotic cells serves to suppress epithelial cell inflammatory responses, and accordingly the deletion of IGF1R in airway epithelial cells exacerbates airway inflammation (284). Macrophage-derived microvesicles may contain anti-inflammatory mediators such as SOCS1 and SOCS3 proteins that were reported to be present within microvesicles released by alveolar macrophages (285). These proteins attenuated JAK-STAT signaling in alveolar epithelial cells during smoking-induced lung inflammation (285). There are undoubtedly many more ways in which phagocytes suppress inflammation, mediate immune tolerance, and orchestrate tissue homeostasis.

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