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Myeloid cell turnover and clearance

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Abstract

Given the dual and intrinsically contradictory roles for myeloid cells in both protective and yet also damaging effects of inflammatory and immunological processes we suggest that it is important to consider the mechanisms and circumstances by which these cells are removed, either in the normal unchallenged state or during inflammation or disease. In this essay we address these subjects from a conceptual perspective, focusing as examples on four main myeloid cell types (neutrophils, monocytes, macrophages and myeloid dendritic cells) and their clearance from the circulation or from naïve and inflamed tissues. While the primary clearance process appears to involve endocytic uptake into macrophages, various tissue cell types can also recognize and remove dying cells though their overall quantitative contribution is unclear. In fact, surprisingly, given the wealth of study in this area over the last 30 years, our conclusion is that we are still challenged with substantial lack of mechanistic and regulatory understanding of when, how and by what mechanisms migratory myeloid cells come to die, are recognized as needing to be removed and indeed the precise processes of uptake of either the intact or fragmented cells. This reflects the extreme complexity and inherent redundancy of the clearance processes and argues for substantial investigative effort in this arena. In addition, it leads us to a sense that approaches to significant therapeutic modulation of selective myeloid clearance is still a long way off.

Introduction

Few, if any, individual cells survive throughout the life of the animal, an observation that sets up the critical concepts of cell lifespan, turnover, removal and maintenance of homeostatic cell numbers. These issues are of special interest for understanding the properties of the myeloid cell lineage, which includes cells such as neutrophils, that may exhibit in the normal naïve adult mammal the shortest lifespan of all, but yet are maintained in relatively constant numbers within the circulation. However our understanding of the underlying mechanisms for myeloid cell maintenance and removal is still substantially limited and also requires reexamination in light of new ideas about the ontogeny, characterization and distribution of the myeloid cells in general. Accordingly, this essay will focus on the concepts and questions that, we argue, are in need of exploration, rather than providing a detailed review of what is a huge literature. By focusing on four of the myeloid lineage cell types, (neutrophils, monocytes, macrophages and dendritic cells) we will also be able to bring to the fore many of the key issues that characterize this set of questions.

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Removal of cells implies cell death and destruction with uptake into phagocytes and subsequent digestion within the endosomes. An exception would be loss at extracorporeal sites such as lung, gut, skin etc where the cells may be removed physically. Various forms of programmed cell death (PCD), often, but erroneously, subsumed under the term “apoptosis”, lead to uptake. Clearly, un-programmed cell death (often called necrosis) can generate dead cells and cell debris that are also generally removed by being engulfed by phagocytic cell engulfment. Key to these processes is the necessary recognition of the dying cell or its constituents by the phagocyte – unique forms of “self-recognition” – that seem at first hand to defy the concepts of self/non-self that underpin how we usually think of “immunity”. In addition, and possibly of significant importance, stimulated cells that are still “living” may also exhibit such recognition signals that lead to their removal while still active (see the section on neutrophils) thus serving a potential regulatory role at the level of whole, living, cells.

This removal by endocytic uptake inevitably puts the emphasis on myeloid cells themselves, especially macrophages, as key instruments of the cell and debris clearance (a legacy of Metchnikov’s phagocyte theories). However, it is increasingly clear that many non-myeloid tissue cell types can, either endogenously or after appropriate stimulation, exhibit these endocytic functions, including the engulfment of whole cells up to 15µm in diameter, i.e. clearcut phagocytosis. This point is also exemplified by the extensive literature on intact apoptotic cell clearance in *C. elegans* carried out by near-neighbor tissue cells in the absence of macrophages. A primitive clearance function would be an obvious requirement for tissue development in multicellular organisms, especially evident in substantial metamorphic alterations at different organizational phases seen in numerous animal groups. Implications from some of the observations noted below emphasize the possible unique elements of these endocytic clearance functions for tissue or inflammatory cells that would be in keeping with their early metazoan evolutionary development. In the context of understanding the life history and functions of mammalian myeloid cells, especially in the normal resolution of inflammatory processes to restore tissue homeostasis, the mechanisms underlying their recognition and removal become critical, and even therapeutically targetable.

Accordingly in this discussion, we will first briefly address general issues of removal of cells and cell debris, which, as noted, also significantly involve a key function of phagocytic myeloid cells. Subsequent sections will focus on the four individual cell types, in each case with an emphasis on general points and concepts that, we suggest, are understudied and ripe for new experimental analysis.

Recognition and removal of cells or of cell debris

Before discussing turnover and removal of the different myeloid cell types, it is relevant to first consider the broader question of how dying, effete or fragmented/disrupted cells are recognized and then ingested. This is a form of self-recognition that, for the most part, does not in itself initiate inflammatory, immunological or defensive responses in the tissues and thus, must be distinguished from more classical innate or adaptive immune system recognition processes. Strikingly, this normal cell removal (including of myeloid cells) is

occurring all the time in very large numbers ($>10^{10}$ per day) in fashions that are essentially immunologically invisible.

Recognition

Extensive studies over the last 20 years have revealed numerous surface alterations that occur during programmed cell death that contribute to recognition and removal of the dying cells. Many of these surface alterations are likely also to extend to cell debris if the cell undergoes disruption, either due to induction of externally induced “necrosis” or post-apoptotic cytolysis (often termed secondary necrosis). A general concept here is that as a consequence of such changes on apoptotic/PCD cells, their removal is usually so efficient that a) they are generally cleared before undergoing secondary necrosis, and b) it is very hard to detect apoptotic cells in tissues unless the clearance mechanisms are disrupted or overloaded.

A key early change during programmed cell death is the cell’s inability to maintain the normal phospholipid asymmetry of the plasma membrane, resulting in the outward exposure of phosphatidylserines (PSs) from their normal location on the inner leaflet (1). Detection of this surface PS is an often-used assay for apoptosis and other forms of PCD and is also one of the most important recognition signals for normal removal of the cells. The PS species themselves may be in the form normally present on the inner leaflet or may become oxidized – leading to a various sets of recognition structures. These may be direct receptors on the engulfing phagocyte (for example BAI1, CD36, some scavenger receptors) or various sets of “bridge” molecules (opsonins) that bind the PS on the dying cell or cell debris (e.g. Protein S, MFGE8) but also link to the surface of the phagocyte to initiate ingestion *via* different sets of receptors (Mer or αv integrins as examples). Notably, such “bridge” molecules may be produced by the phagocytes themselves (e.g. MFGE8, thrombospondin-1) or may be present in the local environment (e.g. Protein S produced by endothelial cells).

It is noteworthy that phospholipid asymmetry of the plasma membrane, and thus the normal inner leaflet distribution of PS is energy dependent (see for example, (2, 3)). PS may be exposed on the outer leaflet as a potential clearance recognition ligand under many different circumstances, including inevitably, when the plasma membrane is disrupted, i.e. during primary or secondary necrosis as well as in extrusion of numerous forms of exosomes, microparticles or even enveloped viruses. A number of specific molecules involved in altering the distribution of phospholipids in/on the cell membrane have recently been characterized (4) as well as mechanisms for maintaining the asymmetry under normal circumstances. In addition to PS exposure, many other molecular and structural changes on the surface of cells undergoing PCD have been described, each potentially also leading to interaction with the phagocyte *via* either direct receptor ligation or relevant bridge molecules. Examples include calreticulin from the dying cell endoplasmic reticulum, externally exposed chromatin and DNA, alterations in surface charge and very likely (but not studied) changes in glycosyl groups (glycocalyx). More complete consideration of these recognition processes is given elsewhere (5–7) but the overall picture indicates high levels of redundancy, further supported by the difficulty of experimentally blocking these systems *in vivo*.

Also implicit in these comments is the expectation that different cell types and different circumstances that render cells effete and ready for removal and/or induce frank PCD or necrosis are likely to generate different combinations and permutations of these recognition structures and removal processes. Unfortunately, this point has not always been considered in the past, leading to studies of cell removal using standardized “apoptotic” or “necrotic” cells that may have only minimal bearing on their removal *in vivo* as well as ignoring the contribution to clearance of various forms of cell “debris”. These concepts apply also to normal myeloid cells that end their effective lifespans in the blood or tissues, and is especially true for cells exposed to inflammatory or toxic environmental processes.

Removal

As noted, the most effective route of cell removal in vertebrates appears to be phagocytosis by various categories of tissue macrophages. Importantly, however, the cells that remove effete cells in the bone marrow are poorly categorized or understood, though also important in clearing excess leukocytes that have developed at this site but which are not called into the circulation. This substantial clearance mechanism in the bone marrow extends beyond removal of myeloid cells to, for example, clearance of extruded nuclear material from developing erythrocytes (8).

Ingestion of intact apoptotic cells (the normal process in homeostatic conditions) into macrophages appears in general to involve a somewhat unique phagocyte process that can be distinguished in a number of ways from “classical” phagocytosis mediated by normal innate or adaptive immunological recognition. The involved signal pathways for the apoptotic cell uptake are highly conserved between *C. elegans*, *Drosophila* and mammals (9–11) and show a significant requirement for the GTPase Rac with, in fact, a balancing suppressive action from the GTPase Rho. It has been suggested that the process represents a version of stimulated macropinocytosis (similarly Rac-requiring and similarly responsive to macropinocytosis inhibitors and generally resulting in early spacious phagosomes and concurrent uptake of surrounding fluid and its contents). In this case the usual 200nm size consideration for macropinocytosis is clearly overcome and there is also a requirement for initial tethering of the target cell to the phagocyte. To emphasize this distinction we have termed the process “efferocytosis”, (see (12, 13) and Figure 1). Not surprisingly, given the variability of the surface ligands and potential signaling responses, this concept of a primary uptake mechanism is likely oversimplified and we have argued that the specific uptake mechanisms, as well as the disposition of the phagosome and its contents are urgently in need of detailed investigation. Similarly, the increasing recognition that uptake of necrotic and disrupted cells (cell debris) is critical to understanding resolution of inflammation and normal tissue homeostasis requires investigation of the underlying mechanisms – to this point substantially understudied.

Neutral, Inflammosuppressive and immunosuppressive responses to cell removal

The massive ongoing uptake and removal of circulating cells (including myeloid cells) is normally immunologically and inflammationally silent (as is the similar daily removal of a massive load of retinal outer segments in the eye (14). Simplistically, this may be because the triggers for uptake (recognition signals) just do not stimulate proinflammatory responses

in the phagocytosing cells. On the other hand in many experimental systems uptake of intact apoptotic cells has been shown to be actively anti-inflammatory and anti-immunogenic. This effect may in turn be mediated by intrinsic blockade of potential inflammatory responses (e.g. inhibition of proinflammatory transcription factors such as NF κ B) or by initiation of anti-inflammatory mediators such as TGF β or IL-10 that can act in autocrine or paracrine fashion to suppress responses of cells in the local environment. Examples of both possibilities abound (9, 11,15, 16). Notably, most of these studies have focused on the effects of apoptotic cells in suppressing *stimulated* inflammatory or immunogenic responses in the macrophages or dendritic cells rather than addressing the importance of mere lack of initiation of inflammatory responses when the phagocytes interact and ingest apoptotic cells. The potential difference here between efferocytosis and the phagocyte uptake of true foreign, or opsonin-coated particles is more evidence of the specialized receptors and signaling involved in efferocytosis as well as in the impact of the actual uptake process on the general response of the ingesting cell. These issues are of substantial importance to host response processes in general and in the questions underlying the initiation of autoimmunity. For example, there are numerous reports of associations between ineffective removal of apoptotic cells and induction of autoimmunity in both animals and man (see below). The implications are that if dying or effete cells are not removed (especially for example circulating granulocytes) they can undergo secondary necrosis, thereby releasing internal constituents such as chromatin or other nuclear contents that can initiate inflammation and immune responses *via* standard recognition and uptake as foreign stimuli, for example involving toll-like receptors. On the other hand, there are increasing reasons to believe that at least some forms of non- or post-apoptotic cell dissolution may in fact generate particles that can also be cleared efficiently without inflammatory or immunological sequelae.

These concepts led to ideas of a contrasting dualism between reactions to apoptotic *versus* necrotic cells and even the teleology of “good” *versus* “bad” cell death. This is unfortunate, since the situation is clearly much more complex and the responses of phagocytes to cells and cell debris are much more nuanced and balanced. More realistically, the complexity and redundancy of ligands on dying cells and receptors on the phagocytes is likely to generate a wide spectrum of effects that vary from nothing more than uptake itself, to induction of anti-inflammatory processes, to frank initiation of inflammation and, in the absence of appropriate immunological tolerance, to autoimmunity. As an example, macrophage responses to ligation of receptors associated with PS recognition *versus* those associated with calreticulin recognition were shown to induce anti-inflammatory *versus* proinflammatory responses respectively (5). In real life however, both PS and calreticulin are likely to be present on a given apoptotic cell (as are many other potential ligands) and a given macrophage likely expresses various receptors either for these, or their bridge molecules, and the capacity for variable inflammatory or anti-inflammatory signaling. The net effects are going to be in balance, and certainly make it extremely difficult to mimic *in vitro*. Additionally, as mentioned earlier, it has to be noted that all apoptotic/PCD cells are not the same. They differ substantially by cell type, mode of PCD initiation, stage of cell death, and the nature of the local environment. This is likely even more true of “necrotic” cells, even when this arises from apoptosis when the cells are not cleared (secondary necrosis) and even more in the case of various forms of cell debris in general. In passing, it

should be noted that most studies of necrotic cells have involved *ex-vivo* dissolution of cells either by sonication, heat or freeze/thaw cycles – processes not commonly seen *in vivo*.

Intracellular fate of ingested cells

Basically, ingested PCD cells and cell debris are effectively and rapidly digested within the endosome and as a consequence, are not commonly visible in tissue sections – i.e. the inability to detect apoptotic or dying cells does not disprove some ongoing level cell removal (note (17) as an example). Macrophages are suggested to mediate rapid phagosomal maturation and content digestion compared to dendritic cells (DC), which may reflect the increased ability of the latter to present antigens to the adaptive immune system. Since DC are also believed to ingest significantly through macropinocytic mechanisms and because this may itself result in slower endosomal maturation and digestive capability, in addition to immunological implications, a careful analysis of not only the uptake processes for dying and degraded cells, but also for their intracellular digestion, would seem to be needed.

Circulating neutrophils, monocytes and dendritic cells

Different cell types within the blood are normally maintained at a relatively constant numbers. Since each have finite lifespans in the circulation, this implies control of source input (the bone marrow in mature mammals) and removal as well as some mechanism for sensing the circulating concentrations. For erythrocytes, the sensor appears to be local tissue oxygenation with erythropoietin serving as a rheostat for bone marrow production and release. In the case of myeloid cells (for the purpose of this essay we will focus on neutrophils, monocytes and dendritic cells) the details are much less clear. The most well studied here are neutrophils, with less known about monocytes, and even less about circulating human dendritic cells.

Neutrophil life span and cell turnover in the bloodstream

Studies in the 1960s using tagged cells, suggested that mature neutrophils in the blood stream have a lifespan measured in hours. While this has recently been challenged and might be extended to days (18) the cells are undoubtedly short lived. A key question for all the circulating myeloid cells is whether their time in the circulation in normal circumstances, i.e. loss from the blood stream, is driven by their emigration into the tissues or clearance in the three main removal organs, liver, spleen and bone marrow. This latter fate also raises a significant question. Do the cells in the circulation undergo apoptosis and are then recognized and removed or are they recognized in some way as old or “effete” and then induced to undergo programmed cell death by interaction with cells such as macrophages in the clearance sites, or even endothelial cells in the vasculature?

Equally challenging is the question of how the circulating numbers of neutrophils are regulated. While certainly fluctuating, including in a circadian fashion, the numbers in normal, non-inflammatory conditions remain within certain limits, and estimates of 10^9 neutrophils/kg are released and removed daily (19). Given the life span, this inevitably means rates of input and output are relatively constant under homeostatic conditions, and implies a sensor of some sort to maintain the consistency. Input into the circulation involves

release of mature neutrophils from stores in the bone marrow and upstream of this, production of the cells from hematopoietic precursors. Various candidates have been proposed for the mechanisms of sensing neutrophil numbers though hard and fast conclusions are lacking.

One significant site of neutrophil removal from the blood is in the bone marrow itself, thereby setting up this as the possible site of sensing and control of the numbers in the blood in a so-called neutrophil “turnstile” driven by the IL-23/IL-17/G-CSF signaling axis (20). Investigation has shown that rhythmic release of neutrophils into the circulation (triggered by cycled exposure to light in mice) is followed by their return to the bone marrow and rhythmic reductions in the size and function of the hematopoietic niche (21). This suggests a rheostat for production that is turned down based on the numbers of returning neutrophils, and as return slows, production responds by ramping up again. The mechanism appears to depend on signals from engulfing bone marrow resident macrophages. Alternatively, other investigators describe the active production of G-CSF by bone marrow macrophages in response to engulfment of returning neutrophils, thereby spurring neutrophil production and release to replace the cells that have just been removed. (22). These data may certainly be reconciled, though as yet, have not been, as representing different phases and signals of the same finely tuned rhythmic process underlying neutrophil homeostasis.

While there is some increasing evidence for constituent migration of at least a few neutrophils into tissues (as well as possibly most eosinophils), nonetheless, in the absence of an inflammatory response (considered below), most of the constant daily removal of neutrophils occurs within the spleen (red pulp), liver (Kupffer cells in the sinusoids) or bone marrow – notably all sites with relative low blood flow, perhaps a requirement for the attendant phagocytes to have enough time to interact with and ingest the target neutrophil. In keeping with this hypothesis, the role for functional β_2 integrins on the neutrophils themselves has been implicated as a requirement for their proper clearance (20).

As far as the role of macrophages in carrying out the actual neutrophil removal, their programming state is critical in determining their capacity for cell clearance. For example, activation of peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) in macrophages, have been shown in response to the engulfment of apoptotic cells, and in turn, their activation leads to expression of downstream targets promoting the uptake and disposal of apoptotic cells (23–25). Recent data show a special role for LXR α /LXR β in neutrophil clearance, and in turn, in the maintenance of neutrophil homeostasis. The LXRs are expressed in bone marrow macrophages in an oscillatory fashion and their expression and activation was shown to follow the wave of returning neutrophils. Genetic mutations of these receptors resulted in loss of the rhythmic modulation of the hematopoietic niche suggesting a role within the neutrophil turnstile (21). Further, genetic loss of the LXRs was also shown to be key to neutrophil removal in the periphery: LXR deficient mice showed increases in circulating neutrophil numbers, slower turnover, and accumulation in the spleen and liver under homeostatic conditions {Hong, 2012 #12}. Equally, perturbation of macrophage populations, either depleting their numbers from bone marrow and spleen (26), or genetically deleting macrophage receptors for the recognition of effete cells (27),

similarly leads to neutrophilia though inflammation inevitably results from these manipulations making assessment of their role in homeostasis difficult.

As noted above, the signals on the neutrophil for removal are still not precisely defined, but are hypothesized to depend on the fate of the neutrophil, as part of its aging, death or activation, rather than stochastic processes (15). Aging murine neutrophils are characterized as having relatively low CD62L and high CXCR4, and appear to interact with bone marrow macrophages leading to their preferential engulfment in comparison to younger neutrophils (21). Whether the aged neutrophils are alive or undergoing death at the time of engulfment, or whether death comes after ingestion has not been fully resolved, but experimental data exist to support both possibilities. As with most cell types, there are numerous possible pathways by which neutrophils undergo programmed cell death (particularly relevant to inflammatory settings discussed below) including standard, caspase-induced apoptosis. *Ex vivo*, without additional stimuli, neutrophils undergo apoptosis relatively rapidly and, as a consequence, expose surface markers *e.g.* phosphatidylserines, that are recognized by phagocytes and initiate their engulfment into phagosomes and eventual digestion. Finally, the unique role of oxidants from the neutrophil NADPH oxidase acting either directly (*e.g.* generation of oxidant-modified phospholipids), or indirectly *via* influencing the form of programmed cell death (see below) deserves consideration in the generation of signals for engulfment.

Monocytes in the circulation

The current concept is that there are two major monocyte types within the circulation. In the mouse these can be distinguished by expression of Ly6C, and in humans most readily by CD14 and CD16. The Ly6C^{low} monocytes are considered to be largely restricted to the intravascular location and have been suggested to play roles of “patrolling” the vasculature (28). The luminal crawling of Ly6C^{low} monocytes on the endothelium requires firm adhesion with LFA-1 (CD11ca/CD18, $\alpha_L\beta_2$) integrin and CX₃CR1 to ICAM1, ICAM2 and CX₃CL1 on endothelial cells (28, 29). On the other hand, circulating Ly6C^{high} monocytes have the ability to extravasate into the tissues, both constitutively and in response to inflammatory conditions, with subsequent ability to either maintain their monocyte characteristics or mature in a number of different ways, including development into monocyte-derived macrophages or monocytederived dendritic cells (30) even without inflammation, as has been noted in the gut, skin and lung (31). Tracer studies using BrdU have shown that monocytes in the circulation have a lifespan of approximately 4 days for Ly6C^{high} monocytes and 11 days for Ly6C^{low} monocytes (31–33). Once again, the triggers for eventual removal of monocytes from the circulation are not clear, whether frank apoptosis, or surface changes leading to recognition and/or stimulation of apoptosis or uptake. Major sites for removal are likely macrophages in liver and spleen as well as phagocytic cells in the bone marrow, but this too requires detailed investigation. The proportion of the migratory Ly6C^{high} monocytes that are lost from the circulation as a consequence of their extravasation *versus* clearance from the bloodstream directly is also not known. Notably, the Ly6C^{high} monocytes which traffic into non-inflamed tissues and bypass macrophage differentiation can subsequently migrate, as monocytes, down lymphatics to lymph nodes, with presumed properties that include local antigen presentation (33). Once in

the lymph nodes, the final disposition of the monocytes, as for other myeloid cells gaining access to lymph nodes *via* the afferent lymphatics may be of some importance and will be discussed in more detail below.

Dendritic cells

Unlike mouse, humans have fully differentiated DCs circulating in the blood, including those of the myeloid lineage. Although many transcriptional and functional parallels between murine tissue and human intravascular DC have been assessed the clearance and turnover rate of human DCs in the blood is currently unknown.

Dendritic cells

DC lifespan in lymphoid and non-lymphoid organs varies depending on the DC subtype and location examined. However, most emphasis to date has been on lymphoid DC in the lymph nodes (34) (34, 35). Nonetheless, BrdU studies have demonstrated that the lifespan of myeloid DC in the skin is approximately 10–14 days, but how generally this applies to other tissues needs to be explored. Tissue resident monocyte-derived DCs were previously hypothesized to require GM-CSF as bone marrow derived DCs *in vitro* require GM-CSF (36). However, GM-CSF deficient mice demonstrated that monocyte-derived TNF α and iNOS producing DCs did not require GM-CSF for development *in vivo* (37). Therefore, what mediators drive tissue trafficking monocytes to either remain as monocytes or differentiate into macrophage-like or dendritic cell like cells is currently unknown. The exact counterpart of this dichotomy is likely common to the human situation though may not be as clearly defined as in mice.

When activated to migrate from their tissue locations into the lymph node it is generally recognized that the DCs are short-lived and undergo apoptosis and removal within 2–3 days. The stimuli/conditions driving this effect again need further investigation. Furthermore, inflammation in the tissue of origin did not substantially prolong the lifespan of migratory DCs within the draining-LN (34). On the other hand, some studies have suggested that upon TLR stimulation Bcl-2/Bcl-xL is up-regulated and DC survival is prolonged. In addition, in the absence of Bcl-2 DC turnover was increased (38). It has been hypothesized that the fast turnover rate of DCs functions to regulate antigen presentation to cognate T cells, ultimately limiting immune activation. Since DCs do not exit LNs via efferent lymphatics, removal occurs within the T cell zone of the LN node, but which cell-type performs this clearance function is unclear – possibly local DC themselves but perhaps more likely, medullary macrophages or stromal cells.

The recent emphasis on studies of parabiotic mice to address issues of myeloid cell origin and turnover suggest that this approach could help answer many of these questions. For example, longer-lived cells (B cells, T cells and Ly6C^{low} monocytes reach equilibrium between both parabiotic partners. However, due to their shorter lifespan neutrophils and Ly6C^{high} monocytes do not attain equilibrium in the blood even after 120 days of parabiosis (35). It was also shown that blood-derived DC precursors (preDCs) are rapidly cleared from circulation (residence time <2 hours) and do not fully equilibrate between the parabionts, i.e. behave similarly to circulating Ly6C^{high} monocytes.

Effects of neutrophil and monocyte cell emigration into tissues and clearance from inflammatory lesions

Neutrophils in inflamed tissues

It has generally been assumed that neutrophils remain in the vasculature until called into inflammatory sites and that they are subsequently removed locally in the inflamed tissues, or if not so removed, remain as a major constituent of pus. Notably, relevant *in vivo* studies of their accumulation and fate are less clear, in part because of the difficulties in distinguishing neutrophil persistence from newly arriving cells in the face of normal efficient clearance of the dying cells. Further, there are increasing suggestions that neutrophils may under some inflammatory circumstances migrate back into the bloodstream (39) as well as down afferent lymphatics to gain access to, and accumulate in, lymph nodes (40). We will consider the issue of clearance of myeloid cells within the lymph nodes below in a separate section. Additional routes of clearance apply after migration onto epithelial surfaces. For example, within the inflamed alveolus, much of the post-inflammatory neutrophil clearance likely occurs through phagocytic uptake and digestion by alveolar macrophages just as in tissues (41) (Figure 2)..

In other instances, neutrophils exit the body and escape phagocytic recycling. An interesting special case is the constant emigration of neutrophils to the mouth through the gingival crevice with removal *via* the saliva (42). Similarly, emigration into the bronchi results in removal by the mucociliary escalator, and emigration through the colonic epithelium results in elimination via the gastrointestinal tract. While critical for host defense at these sites of the body that interface with the external environment, the constant loss of such neutrophils, arguably, in response to inflammatory signals of relatively low intensity, have yet to be quantified or accounted for in overall recycling models.

Importantly, whatever the actual lifespan of neutrophils in the circulation may turn out to be, various forms of inflammatory stimulation of neutrophils appears to delay the “spontaneous” development of their apoptotic pathways. This has mostly been studied *ex vivo*, where onset of “spontaneous” apoptosis without specific added stimuli usually occurs over 18 hours. Stimulation of the cells with pro-migratory or pro-inflammatory stimuli such as chemokines or TLR agonists, extend the time before apoptosis sets in (43). This has been suggested to have functional consequences in both optimizing neutrophil protective activities in the inflammatory process but with potentially negative consequences in disease states where neutrophil lifespan is prolonged and inflammation persists.

Neutrophil apoptosis has been well studied *in vitro* caused by both intrinsic as well as extrinsic signals, and in the important context of phagocytizing microbes (43). Standard caspase-mediated apoptotic signaling pathways, nuclear condensation, exposure of PS and other recognition ligands etc are all evident. Additionally, other signals, some unique to neutrophils and subverted from host defense activities, have also been described (15). Of some interest, neutrophils, though they do release microparticles, do not appear to generate the apoptotic blebs and larger apoptotic bodies that are seen in many parenchymal cell apoptotic processes. Equally, there is some indication that the cells remain intact for longer

in the apoptotic state. Whether these are general features of myeloid cell PCD is not clear, as detailed study in monocytes and macrophages is less extensive. Teleologic suggestions have been raised about the need to maintain cell membrane integrity throughout the clearance process in the face of the potentially injurious contents of these cells and their potential to cause tissue injury. Clearly in circumstances in which removal of apoptotic neutrophils by macrophages is delayed, including a number of chronic disease states (see below), the cells undergo so-called post-apoptotic, or secondary necrosis in which the integrity of the plasma membrane is lost and intracellular contents including a variety of DAMPS and proteases are released.

Of note, other forms of neutrophil programmed cell death are increasingly recognized, each likely resulting in differing signals to the inflammatory milieu and consequences. Neutrophils harboring large vacuoles, likely representing autophagosomes, evident during infections and autoimmunity, are thought to undergo autophagy-associated death (44). Characteristics include mitochondrial swelling, nuclear condensation and intact plasma membranes. Though precise mechanisms are yet to be elucidated, this form of death may represent a branch point from apoptosis in which caspase activation has been inhibited by survival signals, *e.g.* GM-CSF.

In addition, neutrophils undergo a unique form of “death”, termed “netosis” in which the nuclear membrane is disrupted, with the extrusion of chromatin both into the cytoplasm and eventually into the extracellular environment (45). Of note, the original descriptions of this process relied on stimulation of the neutrophils with the phorbol diester, PMA, and criticisms of these observations have been levied accordingly. Recent studies using more physiologic stimuli, have shown that neutrophils can extrude nuclear DNA (mitochondrial DNA extrusion has also been described) while still clearly carrying on the business of being alive; they can still chemotax and actively phagocytose microbes (46). This cellular disruption is hypothesized to play an important role in host defense demonstrated both *in vitro* and *in vivo* (46). In the context of this essay, we clearly need also to think about mechanisms by which these disrupted cells and their contents are ultimately removed, and the inflammatory consequences of signals from their intracellular contents.

Reactive oxygen species almost inevitably play important roles in cell death pathways, and special mention should be made of the role of the phagocyte NADPH oxidase in determining the fate of neutrophils. For instance, activation of the oxidase by TNF α and integrin engagement appears to enhance apoptosis by enhancing mitochondrial membrane permeability, whereas high levels of oxidase activation are implicated in autophagy-associated death as well as netosis (15, 43). Notably, mutations of the NADPH oxidase that render it nonfunctional are associated with prolonged survival of neutrophils (see below). Aside from playing a role in determining the programmed cell death pathway, activation of the NADPH oxidase leads to the production of a modified phosphatidylserine, lysophosphatidylserine. This lipid accumulates both in neutrophils that are activated and fully functional, as well as those aged in culture and apoptotic (47). It signals via a G-protein coupled receptor, G2A on macrophages for engulfment of neutrophils, alive or dead (48, 49).

Monocytes and macrophages

Origins

As discussed elsewhere in this book, the current paradigm indicates two independent sources for macrophages – embryonic progenitors in the fetal liver and yolk sac and bone marrow-derived monocytes. Most so-called “tissue-resident” macrophages derive exclusively from the former, and colonize tissues during embryogenesis (or immediately after birth). Exceptions occur in barrier tissues, namely the lungs, skin and gut where mixed ontogenies may exist. For example, in the lungs alveolar macrophages arise exclusively from embryonic precursors, whereas extraluminal macrophages (often referred to as “interstitial” macrophages) may arise from circulating monocytes (50, 51) or embryonic precursors (33). In comparison, macrophages in the dermis and gut appear to primarily derive from circulating monocytes (52, 53). Whether commensal microbiota play a role in monocyte recruitment and subsequent development at these barrier sites during homeostasis remains unclear. Certainly, during inflammatory processes the recruitment, maturation, programing and fate of monocytes and macrophages changes considerably. Accordingly, in the following paragraphs homeostasis and inflammation will be discussed separately.

Tissue resident macrophages

Classic examples of embryonic-derived tissue macrophages include brain microglia, liver Kupffer cells and alveolar macrophages. These macrophages replicate locally throughout post-natal life, and unless they are depleted or destroyed (e.g. by gamma radiation, clodronate liposomes, etc) they are not replaced by monocytes from the circulation. While the mechanisms that implement and regulate the either the removal or local self-renewal of these macrophages are not known, it is noteworthy that their normal lifespans (i.e. in the absence of overt inflammation) are markedly different. For example, alveolar macrophages in the mouse have been shown to persist for at least 60 days and probably longer (52, 54) whereas resident peritoneal macrophages have considerably shorter life spans (approximately 15 days) (our unpublished observations). The programing states (sometimes termed “phenotype” - though we would argue that this term is misleading) of macrophages in these sites also varies considerably. Critically, both cell turnover rates and programing states are highly influenced by the local environment. To illustrate, the alveolar macrophage exists in an extraluminal compartment where it is bathed in a lipidic film at an air-liquid interface with ambient oxygen tension. In stark contrast, peritoneal macrophages exist in a near anoxic environment that is bounded by the mesothelium. Intriguingly, peritoneal macrophages transferred into the airspaces display cell surface molecules and functional properties characteristic of alveolar macrophages within a week (55).

Thus the general concept of the macrophage as a cell that is highly adaptable to its environment (i.e. exhibiting enormous plasticity) is critical. Whatever the precise signals and processes involved, the environment is clearly paramount in determining macrophage life history. For the moment we must assume that the eventual death and removal of these resident cells likely will involve some form of programmed cell death, but the triggers here for normal turnover are completely unknown, as to a significant degree are the removal processes. A little more is known for monocyte-derived macrophages that accumulate in

tissues during the process of inflammation and thus removal of these cells is discussed in more detail below and contrasted where possible with the resident cell type.

Inflammatory macrophages

During inflammation, mononuclear phagocyte numbers increase dramatically. Two sources account for this – local replication of resident tissue macrophages and recruitment of monocytes from the circulation. As noted above, some of the monocytes that emigrate into an inflammatory site undergo maturational changes that result in their legitimate consideration as macrophages. This includes a huge increase in cell constituent synthesis, resulting a much larger cell, cytoplasm to nuclear ratio, increased numbers of mitochondria, lysosomes, membranes as well as a number of surface markers. As a consequence, in any inflammatory lesion there is a spectrum of cells in various stages of this maturation that are often lumped together under the general term “inflammatory monocytes”. Importantly, the relative contribution provided to the total mononuclear phagocyte pool by resident tissue macrophages versus recruited mononuclear phagocytes varies by anatomic site and is greatly influenced by the nature and magnitude of the stimulus. For example, administration of LPS into the lungs leads to massive recruitment of circulating monocytes yet induces only modest proliferation of resident alveolar macrophages (52). Conversely pleural infection with helminthic organisms drives marked proliferation of resident pleural macrophages but only minimal recruitment of monocytes from the circulation (56). Additionally, as discussed at length elsewhere in this book, the macrophages that exist in inflammatory conditions exhibit a variety of environment-induced programming states that change considerably over time and that are often associated in a contributory way with the progression of inflammation at its early stages and later with its resolution. Critically, as noted in Figure 3, these monocyte-derived macrophages as well as the increased numbers of monocytes accumulated in inflamed tissues, are eventually cleared as the inflammation resolves.

Resolution of Inflammation

As inflammation resolves, tissue macrophage and monocyte numbers return to homeostatic levels. The question of where the “inflammatory macrophages” go is long-standing and only partially answered. In basic terms, three potential fates exist; migration from the site, *in situ* death, and in the case of tissues that contact the external environment (such as the respiratory tract, gut lumen and skin) extracorporeal elimination (e.g. expectoration, sloughing, etc). As described above, monocytes clearly have the ability to migrate from tissues using the lymphatics as a conduit (33). However, whether monocytes can also exit tissues by direct egress back into the bloodstream remains unclear. That Macrophages emigrate from the peritoneum *via* the lymphatics has also been reported (57, 58). As mentioned above, monocyte maturation occurs across a continuum and it is likely that at least some of these emigrating macrophages represent inflammatory monocytes that have gained cell surface molecules typically associated with macrophages. *In situ* death of macrophages and subsequent efferocytic removal represents a clear mode of exit, and in some instances provides a critical means of host defense by eliminating ingested pathogens. Notably, in the peritoneum and lungs, macrophage death appears to be the dominant mechanism for macrophage elimination during resolving inflammation, with lymphatic egress playing only a minor role (52, 59). As mentioned above, numerous pathways for

death exist, including apoptosis, autophagy, necroptosis and frank necrosis – all of which may be operative in driving elimination of macrophages from inflammatory sites. Critically, both the mode of death and the frequency with which cells die are likely to be influenced by the local environment tissue-specific factors, and perhaps even the origin of the cell itself. In this regard, studies of resolving inflammation in the peritoneum (59) and lungs (52) demonstrate that recruited monocyte-derived macrophages undergo programmed cell death, while at the same time resident macrophages (i.e. embryonic derived macrophages) persist – even though both populations of cells exist in the same environment.

The developing theme of fundamental (transcription factor dependent) differences between embryonic derived self-renewing “resident” macrophages and the monocyte derived inflammatory macrophages under consideration here, may in fact be somewhat blurred and in need of more investigation. Thus, although study of resident alveolar macrophages shows their considerable ability to survive throughout the course of an acute inflammatory reaction, there is nonetheless some loss of these cells that are eventually replaced, in part apparently from a proportion of the monocyte-derived cells (52). Intriguingly, gene expression comparison of these, *versus* the persistent original “resident” macrophages shows that over time the monocyte derived cells became almost identical with the resident cells, differing in only a few genes (which did include some of the relevant transcription factors) (unpublished or some other attribution?). This observation, which is likely mirrored at other sites, such as the peritoneum, makes a strong case for the influence of the environment in molding the character of the local macrophage, whatever its proximal origin.

The frequency of apoptotic macrophages that can be detected in resolving inflammatory lesions is generally low, suggesting their rapid removal and digestion. However, the question of “Who clears the dying macrophages?” remains largely unanswered. Both the remaining macrophages and non-professional phagocytes, such as epithelial cells have the capacity to engulf dying cells, though quantitative studies describing their relative contributions are lacking. The answer will almost certainly be organ/site specific and may also vary with the inflammatory stimulus. In any case, as described above for neutrophils, the process appears to involve exposure of “eat me” signals on the dying cell’s surface. Again, phosphatidylserine (PS) most likely plays a major role, although additional apoptotic-cell associated molecular patterns (ACAMPs) including changes in surface charge and glycosylation, and exposure of calreticulin on the cell surface may also be operative. Notably, activated macrophages can express PS on their surfaces, even when they are not apoptotic (60), thereby suggesting that PS exposure alone is insufficient to drive engulfment and that loss of “don’t eat me” signals (e.g. CD47, CD31) on the surface of the dying cell may also be required. The many molecules used by macrophages to recognize dying cells are described in detail elsewhere (5–7). However, it must be noted that the most of our knowledge derives either from studies performed in the steady state (as described above) or during the acute neutrophilic phase of inflammation. The local environment changes significantly as inflammation resolves, and it is therefore likely that phagocytic receptor expression and uptake mechanisms are also likely to change.

Disposition of myeloid cells migrating to the lymph nodes

One central theme in the removal of a number of myeloid cell types from tissues is migration/clearance down the lymphatics. Thus as noted, this appears to be one mode of removal of macrophages from the inflamed peritoneum and is clearly relevant for disposition of tissue migratory DC or constitutively migrating monocytes. However, this process only kicks the can down the street. Are the cells then removed in the local lymph node? Do some even pass on down the chain to further lymph nodes or even into the blood stream *via* the lymphatic ducts. We suggest that this last pathway for removal seems unlikely since mature macrophages are of substantial size and will not pass readily through capillary beds. Basically, programed cell death and local uptake by phagocytes in the lymph nodes seems most likely for this secondary process of clearance from the tissues. As noted above, the life span of migratory myeloid DC that have reached the lymph node is no more than a few days. However, reflecting a constant theme in this essay, the precise stimuli that initiate this, and the cells (macrophages, stromal cells, etc) and recognition processes involved in their subsequent phagocytic removal have not been clearly defined. Nonetheless, we must suppose this to be a quantitatively substantial clearance process with significant relevance for maintenance of tissue homeostasis.

Defects and Disease

To no great surprise, systems as essential to normal development and homeostasis are not immune from inherent or induced defects and these can include loss of function, as well as gain of function effects. In the case of myeloid cells, their additional role in the removal of damaged cells and debris leads to potential defects in both their own removal as well as their ability to mediate the removal process. A few examples will suffice to illustrate these points, but one can expect many others and a significant contribution to a wide variety of disease processes.

Defects in removal of inflammatory myeloid cells. Chronic Obstructive Pulmonary Disease (COPD)

In any inflammatory condition, inability to mediate the rapid and highly efficient removal of the accumulated neutrophils and monocytes would be expected to, and does, prolong the inflammation. Such effects can be caused by the effects of stimuli to the inflammatory cells that block their normal induction of programed cell death as well as in defects of the removal process itself. As noted above, neutrophil apoptosis can indeed be delayed by a host of potential inflammatory mediators as well as by stimulation of pattern recognition molecules from external or host-derived sources. We bring up COPD in this context because the long-term destruction of the lung tissue underlying this extremely common and important disease has been suggested to result from persistent low-grade inflammatory, especially neutrophil-driven, injury. Cigarette smoke, either directly or indirectly in a feed-forward inflammatory mediator-induced fashion can prolong the life of neutrophils leading to accumulation in the lung tissue. However, it has additionally become apparent that COPD is associated with defects in the ability of macrophages in this tissue to recognize and remove apoptotic cells (i.e. the neutrophils), thereby adding an additional component to persistence

of inflammation and its contribution to tissue destruction (61–63). Fundamentally, this example leads one to raise a challenge to the general possibility of similar feedback loops contributing to many forms of chronic inflammation, whether the targets are neutrophils selectively, or inflammatory myeloid (or lymphoid) cells more generally. In each case the mechanisms underlying the defects may be different, and may be either external or internal (or both), but, we would argue, are worth further exploration in the hope of ameliorating the ongoing process.

Chronic Granulomatous Disease, CGD

Another example, in this case with a genetic underpinning, is CGD. Various defects in subunits of the NADPH oxidase lead to loss of the oxidative burst (particularly relevant in neutrophils) with concomitant defects in bacterial and fungal host defense. However, in addition to problems with constant infections, the patients also exhibit persistent inflammatory and granulomatous problems (hence the name). Relevant to the current discussions, the defective neutrophil NADPH oxidase results in lack of normal changes on their surface during inflammation such as generation of lysophosphatidylserine that usually render the cells more palatable for uptake and removal by macrophages (48, 64). In addition, the genetic defects are also associated with altered programming of the macrophages towards decreased ability to ingest apoptotic cells. Consequently, these two effects act together to result in defective neutrophil clearance, and thus, persistent inflammation. Notably, the effect is seen most clearly in the context of inflammation, i.e. when the NADPH oxidase is normally activated, and to a much lower extent, at the level of normal neutrophil turnover in the blood.

Autoimmune processes including Systemic Lupus Erythematosus (SLE)

An intriguing observation that pervades the studies of mechanisms and extent of apoptotic cell clearance is that defects in these processes, for example loss of key receptors for apoptotic cell recognition and uptake, are frequently associated with generalized autoimmune disease. Examples include mice lacking the scavenger receptor CD36, bridge molecules MFG-E8 and C1q, Mer tyrosine kinase, and G-protein-coupled receptor kinase 6 (GRK6, (65)) which normally enhances apoptotic cell engulfment through Rac1 activation. When coupled with observations of defective apoptotic cell clearance in human systemic lupus erythematosus (SLE) the hypothesis that inefficient clearance, of for example dying neutrophils, results release of intracellular constituents such as nuclear contents that can then initiate autoimmune responses (66). Note also that CGD patients and mice, again showing inefficient clearance of neutrophils, are also prone over time to develop autoimmunity (67, 68). The extent to which defects in normal cell removal are generally associated with autoimmune responses and the additional controls that may serve to limit this extent are of considerable interest and potential importance.

Hemophagocytic lymphohistiocytosis (HLH)

In the other direction, a group of syndromes often termed HLH is associated with *increased* phagocytic uptake and removal of blood cells, including myeloid cells, throughout the body (69, 70). These can be either developmental or associated with viral infections and are accompanied and presumably induced by very high levels of systemic cytokines, particularly

interferon gamma. Specific mechanisms by which the leukocytes (and erythrocytes) are altered or become recognizable by the phagocytes are not clear. The macrophages involved in the ingestion are likely substantially over stimulated by the cytokine storm, but whether the loss of normal discrimination against uptake of blood leukocytes is solely at the level of the targets or also in the functions of the phagocytes is also unclear. While the clinical conditions themselves are rare, and animal models are limited (69) full exploration of the underlying mechanisms would seem to have potential for general understanding of the controls normally involved in leukocyte maintenance and removal.

Implications and conclusions – maintenance of homeostasis

We are consistently impressed by the extraordinary variety of forms and mechanisms by which cells can be induced to undergo programmed death, variety that is certainly manifest by the myeloid lineage as well as within other cell categories. This extensive repertoire extends also to the changes on the cell surface, or on cell debris that results, leading to recognition by phagocytes and mediating their removal. Increasingly it is also becoming apparent that these different ligands likely stimulate many different uptake mechanisms in the phagocytes. A sense of enormous redundancy results, suggesting the substantial importance to a multicellular organism of being efficient in its ability to remove cells no longer needed or undergoing aging, damage, abnormal function or death i.e. to maintain homeostasis. On the other hand, it also emphasizes the need consider each cell type, situation and environment separately, and explicitly, where possible, to study the removal processes *in situ* in the relevant environment *in vivo*.

Not surprisingly, increasing understanding of the processes of myeloid cell removal has led to consideration of therapeutic approaches to its enhancement, for example in inflammation. Given the complexities noted above, these approaches are in their infancy and fraught with not only the redundancy problem, but also contrasting concerns relating to the balance between protective *versus* damaging effects of inflammatory leukocytes. We note only two potential examples here (of many) to illustrate the possibilities as well as issues involved. In our own studies, stimulation of PPAR γ with pioglitazone is showing intriguing promise in overcoming some of the defects in neutrophil removal seen in chronic granulomatous disease by altering both the palatability of the neutrophils for uptake as well as the effectiveness of their uptake by the macrophages (unpublished observations). In another example, a flavone (wogonin) inducer of eosinophil apoptosis was recently reported to have attenuating effects on airways inflammation and allergic responses (71, 72). Other areas of investigation that would seem appropriate to this discussion and that have also perhaps not received the attention that would now be due, are the effects of aging of the animal and human on the clearance processes and at a cellular level, the effects of senescence of the cells (or precursors) themselves. In the case of the myeloid cells, this could apply both to the cells destined for removal as well as those doing the removal itself.

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Figure 1. Mechanisms for recognition and uptake of apoptotic cells

Changes on the apoptotic cell surface, including exposure of phosphatidylserine and other normally internally located molecules are recognized by surface receptors on the phagocyte leading to tethering of apoptotic cell and transduction of uptake signals. Bridge molecules (opsonins) in the environment or produced by the phagocyte may also recognize the apoptotic surface changes and also a different set of receptors on the phagocyte to initiate tethering and/or signaling. These processes are significantly redundant and also highly regulated, by enhancing or inhibitory stimuli. Viable cells may also avoid removal by expressing “don't eat me” stimuli that block the recognition and/or uptake processes.

Figure 2. Uptake of apoptotic neutrophils by a macrophage in the alveolar air space during resolution of inflammation in the lung

Figure 3. Time course of a standardized acute inflammatory response in the lung showing accumulation and removal of neutrophils and monocyte/macrophages

The resident alveolar macrophages persist throughout the inflammation and do not substantially change in numbers. Some of the recruited Ly6C_{high} monocytes mature into macrophages (and DC) and some remain as monocytes. The macrophages undergo a variety of programming changes during the course of the inflammation participating in first its initiation and then its resolution. The monocytes and macrophages are cleared as the inflammation wanes, mostly by undergoing PCD and engulfment, though some of the monocytes migrate to the local lymph nodes and, at this site, some of the cells may also be cleared physically up the airways by the mucociliary escalator.