Innate immune perturbations, accumulating DAMPs and inflammasome dysregulation: a ticking time bomb in ageing

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Abstract

Ageing has pronounced effects on the immune system, including on innate immune cells. Whilst most studies suggest that total numbers of different innate immune cell populations do not change dramatically during ageing, many of their functions such as phagocytosis, antigen presentation and inflammatory molecule secretion decline. In contrast, many endogenous damage-associated molecular patterns (DAMPs) accumulate during ageing. These include reactive oxygen species (ROS) released from damaged mitochondria, extracellular nucleotides like ATP, high mobility group box (HMGB) 1 protein, the receptor for advanced glycation end products, oxidized low density lipoprotein, amyloid-beta (Aβ), islet amyloid polypeptide and particulates like monosodium urate (MSU) crystals and cholesterol crystals. Some of these DAMPs trigger the activation of inflammasomes, cytosolic danger sensing signalling platforms that drive both the maturation of specific pro-inflammatory mediators such as IL-1β, as well as the initiation of pro-inflammatory pyroptotic cell death. Herein, we review the evidence that dysregulated inflammasome activation, via altered innate immune cell functions and elevated levels of DAMPs, contributes to the establishment of chronic, low-grade inflammation (characterized by elevated levels of IL-6 and C-reactive protein) and the development of age-related pathological processes.

Highlights

- Many functions of innate immune cells decline during ageing.
- Certain DAMPs and pro-inflammatory mediators increase during ageing.
- Ageing may be linked with dysregulated inflammasome responses.
- Controlling inflammasome activation may limit age-related pathologies.

Keywords: Autophagy, DAMPs, inflammasome, inflammation, Nod-like Receptors, Pattern Recognition Receptors

1. Introduction

Links between ageing and health decline have provided an enduring fascination throughout human history. The Greek philosopher Aristotle (384-322 BC) held the view that the human body had a specific amount of heat that was lost with age, and thus ageing brought vulnerability to illness. The Roman physician Galen of Pergamum (131-201 AC) considered that ageing was not a disease, but rather a peculiar state between health and illness (Grant, 1963). The relationship between ageing and health deterioration still intrigues many modern day researchers who are now focused on identifying causative molecular mechanisms. It is well known that susceptibility to infectious diseases increases with age, as does the severity of the associated pathology. For example, there is a higher prevalence of death due to infection, particularly pneumonia and viral infections, in elderly people as compared to younger individuals (Blot, et al., 2009, Marston, et al., 1997, Thompson, et al., 2003). Indeed, in the USA, 90% of patients who die from pneumonia are over the age of 65 years (Mouton, et al., 2001). Perturbations in immune functions are likely to be central to age-related infectious disease susceptibility and severity, and indeed, immune dysregulation is thought to contribute to many age-related illnesses. Of interest in this regard is the innate immune system, which not only provides the first line of defence against infectious diseases, but also drives much of the pathology in chronic diseases.

Integral to innate immune cell activation are germline-encoded pattern recognition receptors (PRRs) that sense danger in the form of exogenous microbe-associated molecular patterns (MAMPs) and endogenous damage-associated molecular patterns (DAMPs) (Kumar, et al., 2011). Some PRRs, particularly certain members of the Nod-like Receptor (NLR) family of PRRs, form a cytoplasmic signalling complex called the inflammasome in response to specific MAMPs and DAMPs. Activation of this signalling pathway results in both the release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18, as well as the

initiation of pyroptotic cell death. In this review, we provide an overview of the impact of ageing on the functions of different innate immune cell populations. Specifically, we review the evidence linking ageing to a decline in certain innate immune functions, as well as elevated levels of specific DAMPs. We propose that these changes contribute to dysregulated inflammasome function, which drives chronic, low-grade inflammation that is characterized by elevated levels of specific inflammatory mediators such as IL-6. This sustained inflammatory state in turn contributes to the development of age-associated pathologies (**Figure 1**).

2. Effects of ageing on cellular and soluble mediators of immunity

2.1 Effects of ageing on acquired immunity

Age-related defects in immunity could result from perturbations in the numbers and/or functions of specific immune cell populations. Studies that have analyzed the white blood cell composition of elderly people suggest that, although the total number of leukocytes does not vary dramatically, ageing does affect numbers of specific immune cell populations (Desai, et al., 2010) (**Figure 2**). For example, lymphocyte numbers decline with age and, in mouse, this is linked to a reduction in B cell output from bone marrow (Miller and Allman, 2003), as well as a diminution in the naïve T cell output from the thymus (Linton and Dorshkind, 2004). In parrallel to this decline in naïve lymphocytes, the proportion of memory T cell populations increases in the tissues and the bone marrow (Herndler-Brandstetter, et al., 2012, Kovaiou, et al., 2005). This may be driven, at least in part, by persistent viral infections (Salam, et al., 2013). As a consequence, the reduced number of naïve T cells and reduced T cell receptor diversity leads to increased susceptibility to infections from new pathogens, an impaired ability to respond to new infections and a diminished response to vaccination (Nikolich-Zugich, 2008). Moreover, the accumulation of memory lymphocytes can

predispose to the development of autoimmune diseases (Goronzy and Weyand, 2003). Not surprisingly then, much of the literature on immune function during ageing focuses on acquired immunity. In contrast, we focus this review on changes in innate immunity during ageing. Indeed, lymphocyte perturbations may be a consequence, in part, of age-related dysregulation in innate immune cell functions such as cytokine production, antigen presentation and inflammasome function.

2.2 Age-related changes in innate immune cell populations and their functions

Ageing affects numbers and/or functions of certain populations of innate immune cells. Mast cells play key roles in responses to allergens and large extracellular pathogens, and also regulate wound healing through their capacity for IgE-dependent release of potent inflammatory mediators such as histamine and tumour necrosis factor (TNF). The number of mast cells is reduced with age (Asboe-Hansen, 1968), and a recent study (Tsuboi, et al., 2012) suggests this decline is due to inefficient replenishment. Reduced expression of c-Kit, the receptor for stem cell factor, was also observed in 10 week old mice as compared to 6 week old mice, and this correlated with a reduction in numbers of dermal mast cells (Hart, et al., 1999). Since the stem cell factor/c-Kit axis is essential for mast cell development (Okayama and Kawakami, 2006), this provides one possible explanation for reduced mast cell replenishment. The functions of mast cells also appear to alter with age. In contrast to mast cells from young mice, those from old mice responded robustly to prostaglandin E2, with more pronounced prostaglandin E receptor-dependent degranulation (Nguyen, et al., 2005). The authors thus proposed that as an organism ages, mast cell sensitivity to specific inflammatory mediators increases.

Like mast cells, eosinophils also contribute to parasite control and allergen-induced pathology. However, there is less evidence of perturbations in these cells during ageing. For example, no variation in eosinophil numbers in the sputum of asthmatics was found between young and elderly patients (Vignola, et al., 2003). However, one study did report a significant decrease in IL-5-mediated degranulation in peripheral blood eosinophils from elderly asthmatic patients (average age 67 versus 30 years) (Mathur, et al., 2008). The same study also showed that other functions, such as superoxide production, adherence and chemotaxis, were not altered.

In contrast to the lack of evidence for eosinophil perturbation, substantial evidence links ageing with effects on neutrophils, granulocytes that are the most abundant leukocyte population in human blood. Although no differences were observed between young and elderly people in neutrophil numbers or granulocyte colony-stimulating factor-induced neutrophil production (Price, et al., 1996), various neutrophil functions are reported to be altered during ageing. For example, neutrophil phagocytic capacity was lower in the elderly (>65 years) than in younger subjects (23-35 years), as was expression of CD16/Fc gamma receptor III (Butcher, et al., 2001). Importantly, antimicrobial functions of neutrophils from elderly people were also reported to be modified; reactive oxygen species (ROS) production after *Staphylococcus aureus* challenge was reduced, as was intracellular bacterial killing capacity (Di Lorenzo, et al., 1999, Wenisch, et al., 2000). Neutrophils can expel neutrophil extracellular traps (NETs), which are composed of chromatinized DNA and antimicrobial proteins, to immobilize and destroy extracellular pathogens. Impaired NET formation was reported in neutrophils from elderly donors (Hazeldine, et al., 2014). As NET formation is dependent upon NADPH oxidase activity (Fuchs, et al., 2007), this effect could be another consequence of decreased ROS production. Similarly, Tseng et al (Tseng, et al., 2012)

showed that old mice infected with *S. aureus* displayed impaired NET production, low production of certain chemokines (CXCL1, MIP-2 and KC), and reduced neutrophil recruitment to the site of infection. Reduced chemotaxis of neutrophils from healthy older subjects was also described by Sapey et al (Sapey, et al., 2014). In this case, the effect was linked to the basal activation state of phosphoinositide 3-kinase. Interestingly, inhibition of this kinase restored efficient chemotaxis. Reduced production of chemokines such as IL-8, as well as other defects in cell functions, have also been linked to reduced neutrophil expression of Toll-like Receptor (TLR)1 in adults over the age of 65, as compared to those from adults aged between 21 to 30 years (Qian, et al., 2014). Thus, many important neutrophil functions deteriorate during ageing.

Through their capacity to recognize absent or altered major histocompatibility complex (MHC) class I expression, natural killer (NK) cells can detect and destroy virus-infected or tumor cells. Age-associated changes in NK cells in the elderly have been extensively documented by Mocchegiani et al (Mocchegiani and Malavolta, 2004). One of the major differences is that the elderly reportedly have an increased number of total NK cells in their blood (Plackett, et al., 2004). More precisely, there is an increase in the $CD56^{-}CD16^{+}$ subpopulation, but a decrease in the $CD56^{bright}$ subset (Le Garff-Tavernier, et al., 2010). Nonetheless, this increase in total NK numbers is counterbalanced by a decrease in the cytotoxic activity per cell (Campos, et al., 2014). This suggests that the increase in number may actually reflect a regulatory mechanism that is attempting to restore appropriate NK functions. Declining NK cell function likely relates to lower levels of perforin (Rukavina, et al., 1998), as well as reduced production of interferon (IFN)-γ, chemokines and ROS (Borrego, et al., 1999, Di Lorenzo, et al., 1999, Mariani, et al., 2002, Solana and Mariani, 2000). These age-related perturbations in NK cell functions may contribute to several age-

related diseases. For example, impaired NK functions in the elderly have been associated with a higher incidence of infection (Ogata, et al., 2001). Similarly, patients with acute myeloid leukemia under the age of 65 years had reduced expression of CD226 (DNAM-1) on their NK cells, as compared to those of healthy subjects (Sanchez-Correa, et al., 2012). As CD226 is involved in the activation of NK cell-mediated cytotoxicity, older subjects may be at risk of having less efficient anti-tumor immune responses. These examples highlight that NK cells from the elderly can display similar phenotypes to those of some cancer patients, which might contribute to impaired tumour surveillance by these cells. Crosstalk between splenic dendritic cells (DC) and NK cells from C57BL/6 mice was also altered with ageing. DC from 80 to 120 week old mice could not activate NKs from either young or old mice, whereas those from 8 to 12 week old mice could efficiently trigger CD69 expression and IFN-γ production by NK cells (Guo, et al., 2014). Thus, impaired DC functions may contribute to a decline in NK cell functions during ageing. In view of the clear links between ageing and NK cell function, future studies may well identify differences in numbers or functions of other innate-like lymphocytes such as ILC22, ILC17 and/or type 2 ILC (Hwang and McKenzie, 2013) during ageing. This seems plausible given that these cells are at the cross-roads between innate and adaptive immunity, and have been linked with various autoimmune and inflammatory diseases (Monticelli, et al., 2012, Sonnenberg, 2014), conditions that are commonly found in elderly people.

Monocytes account for approximately 5% of the white blood cell population. These cells can differentiate into macrophages and DC to replenish certain tissue resident populations during homeostasis and inflammation. There is no clear consensus on the effects of ageing on DC subsets. Some have reported a decrease in numbers of myeloid DC, but not plasmacytoid DC (pDC), in the elderly (Della Bella, et al., 2007). Others reported that numbers of pDC, but not

myeloid DC, declined with age and that only frail elderly people showed a decrease in their myeloid DC numbers (Jing, et al., 2009, Perez-Cabezas, et al., 2007). These reports are of particular interest, given that pDC are major producers of type I IFN, and that type I IFN negatively regulates inflammasome responses (see Section *4.3*). Although monocyte numbers do not appear to be substantially altered in the elderly, the non-classical $CD14^{+}CD16^{+}$ monocyte subpopulation was reported to increase significantly during ageing (Seidler, et al., 2010). Importantly, recent studies have documented that tissue macrophage populations can also arise independently of monocytes. During embryogenesis, embryonic phagocytes seed specific tissues to provide the precursors for several tissue-resident macrophage populations in the adult, under homeostatic conditions (Guilliams, et al., 2013, Perdiguero, et al., 2014, Schulz, et al., 2012). Nonetheless, under conditions of stress as occurs during infection, inflammation and ageing, inflammatory monocytes contribute substantially to the pool of tissue-resident macrophages (Ginhoux and Jung, 2014, Zigmond, et al., 2012). Thus, functional differences between embryonic phagocyte-derived versus monocyte-derived macrophages may contribute to altered tissue macrophage functions during ageing.

Numerous studies have documented age-related perturbations in macrophage functions, including phagocytosis, cytokine production, regulation of wound healing and antigen presentation. Phagocytotic activity of Kupffer cells (tissue resident macrophages of the liver) and peritoneal macrophages was reported to be reduced in aged rats (Izgut-Uysal, et al., 2004, Sun, et al., 1998). However, not all macrophage populations share this phenotype. Another study showed that peritoneal macrophages from aged mice also had impaired phagocytic activity, whereas this was not the case for bone-marrow derived macrophages (Linehan, et al., 2014). Similarly, Mancuso et al (Mancuso, et al., 2001) showed that, in aged rats, phagocytic activity of recruited polymorphonuclear leukocytes was impaired, whereas

that of resident alveolar macrophages was actually substantially increased. Given that recent evidence suggests that a substantial proportion of resident alveolar macrophages are derived from embryonic phagocytes (Guilliams, et al., 2013), differential effects of ageing on phagocytic capacity between macrophage populations could reflect differences in ontogeny (e.g. embryonic- versus haematopoietic stem cell-derived).

In addition to changes in phagocytic capacity, ageing generally leads to reprogramming of macrophage responsiveness to inflammatory stimuli. For example, macrophages from aged mice produced less lipopolysaccharide (LPS)-induced IL-6, TNF, IL-1β and IL-12, which was linked to an elevation in LPS-induced IL-10 production (Chelvarajan, et al., 2005). In contrast, another group linked the age-related impairment of LPS responses in macrophages from C57BL/6 mice to reduced protein expression of CD14, a TLR4 co-receptor (Vega, et al., 2004). Interestingly, the surface expression of TLR4 on macrophages did not vary when comparing peritoneal cells from young (2-3 months) and aged BALB/c (> 18 month) mice (Boehmer, et al., 2004), whereas it was decreased in aged C57Bl/6 mice (Renshaw, et al., 2002). Whilst the findings from these studies cannot be directly compared, it does raise the possibility that genetic background may impact on immune phenotypes during ageing. In human monocytes, differences in cellular responses from young (21 to 30 years) versus elderly (>65 years) donors depended on the specific TLR being triggered (van Duin, et al., 2007). A strong age-associated impairment in TLR1/2-induced TNF and IL-6 production was evident, whereas other TLR-induced cytokine responses (including TLR2/6) remained intact. The authors proposed that decreased surface expression of TLR1 was causal for this, which is consistent with more recent findings in neutrophils (Qian, et al., 2014). Reprogrammed macrophage cytokine secretion profiles during ageing may also have relevance to immunosuppression during cancer progression (Jackaman and Nelson, 2014). Recent work

from Nelson and colleagues (Jackaman, et al., 2013) showed that macrophages from aged mice show an M2-skewed phenotype and produce higher levels of TGF-β in response to IL-4. In addition, only macrophages from aged mice produced IL-4 in the presence of lung carcinoma tumor cell-derived supernatants. This would suggest that tumour-associated macrophages in elderly patients may have a more pronounced immunosuppressive phenotype. Finally, macrophages from young but not old mice could promote IFN-γ production from T-cells, but targeting macrophages from aged mice with IL-2/CD40 could rescue T-cell production of IFN-γ (Jackaman, et al., 2014). Thus, immunomodulation strategies could potentially overcome the immunosuppressive phenotype of tumour-associated macrophages in the elderly.

Resident macrophages play an important role in skin homeostasis and wound healing. Thomas et al (Thomas, 2001) reported an age-associated decrease in wound repair in the skin in both human and mouse. This was linked to a delay in most steps of the wound repair process: cellular infiltration, collagen deposition, angiogenesis and re-epithelialisation. Intriguingly, this healing delay could be overcome by administration of peritoneal macrophages isolated from young mice, whereas macrophages from old mice did not improve healing (Danon, et al., 1989). This effect could be partially explained by an age-related decrease in the production by macrophages of vascular endothelial growth factor (Swift, et al., 1999), a key angiogenesis factor required for wound healing. More recent work has shown that efficient wound healing could be restored in aged mice by using activated mesenchymal stem cells, and that this effect was dependent on macrophage activity (Lee, et al., 2013).

Finally, antigen presenting capacity of myeloid cells is impaired in the elderly. For example, when used as antigen presenting cells (APC), thioglycollate-elicited peritoneal macrophages from aged C57BL/6 mice (18–26 month old) required more time to induce $CD8^+$ T cell clonal expansion and induced lower levels of IFN-γ secretion, as compared to macrophages from 8–12 week old mice (Donnini, et al., 2002, Plowden, et al., 2004). This effect was solely dependent on the APC, as T cells from aged mice showed no defect when the APC were derived from young mice. At least one mechanism that could account for impaired antigen presentation capacity is an age-related decline in IFN-γ-stimulated expression of MHC class II in macrophages (Herrero, et al., 2001), at least in the C57BL/6 mouse. Another study showed that levels of MHC I and II on macrophages from young versus old BALB/c mice were not significantly different, again raising the possibility that genetic background influences immune perturbations during ageing. There is currently limited data on the effect of ageing on antigen presentation in humans. One study found that *in vitro*-derived DC from young (<30 years) versus old (>65 years) individuals did not differ in phenotype or functional capacity, although increased DC numbers were consistently generated from the older individuals (Steger, et al., 1996). However, whether these findings with *in vitro* differentiated DC reflect *in vivo* functions of DC during ageing is an open question.

2.3 Age-related increases in soluble innate immune inflammatory mediators

The general aged-associated hyporesponsiveness of innate immune cells, particularly macrophages, contrasts with the increased levels of certain pro-inflammatory mediators, such as IL-6, IL-1β, IL-18 and TNF, in the blood and/or specific tissues in the elderly. High levels of IL-6 at homeostasis in the elderly are such a consistent finding that IL-6 has been described as the "cytokine for gerontologists" (Ershler, 1993, Ershler and Keller, 2000). Serum concentrations of IL-6 also correlate with disease severity for some inflammatory

diseases, for example in sepsis (Damas, et al., 1992). Therefore, IL-6 might be viewed as a marker of declining health, either through illness or ageing. Indeed, elevated levels of IL-6, as well as C reactive protein (CRP), correlated with mortality in a study of more than 1200 healthy participants over the age of 65 years (Harris, et al., 1999). The value of other inflammatory mediators such as TNF as predictors of health status is still unclear. For example, a study by Bruunsgaard et al (Bruunsgaard, et al., 2003a) reported that, in the case of centenarians, TNF serum levels were a prognostic marker for mortality. However, similar work from the same author found that, in a cohort of 80 year-old people, TNF levels correlated with mortality after 6 years only in males, whereas IL-6 was a good mortality marker in both males and females (Bruunsgaard, et al., 2003b). More recent work from Giovanni et al (Giovannini, et al., 2011) confirmed that TNF, IL-6 and CRP levels were associated with a significantly increased risk of death in the elderly, but TNF concentrations was no longer significant when adjusting for confounding effects such as age, sex and clinical variables. Another study reported that the combination of high levels of IL-6 and low levels of insulin-like growth factor increased the risk of disability and death in elderly women (Cappola, et al., 2003). Not surprisingly, this suggests that combinatorial perturbations of biological pathways impact age-associated pathologies. Yet another inflammatory mediator linked with declining health during ageing is macrophage inhibitory cytokine-1 (MIC-1/GDF15), a cytokine involved in inflammation and healing. A recent analysis of a cohort of 876 male subjects from 35 to 80 years old, correlated MIC-1 serum levels with all-cause mortality (Wiklund, et al., 2010).

Whilst the correlation between declining health during ageing and increased levels of certain inflammatory mediators such as IL-6 has been appreciated for many years, there is growing interest in the contribution of the inflammasome-dependent cytokines IL-1β and IL-18 in

age-associated pathology (Dinarello, 2006). This is underscored by the recent finding that activation of nod-like receptor pyrin-containing domain (NLRP) 3 was linked to systemic, low-grade, age-associated "sterile" inflammation (see *Section 4.1*), leading to the production of IL-1β (Youm, et al., 2013). Importantly, the authors found that animals lacking either Nlrp3 or the IL-1 receptor were partially protected against age-related functional decline. There is certainly evidence for increased levels of IL-1β and/or IL-18 in certain age-related conditions. For example, high serum levels of IL-1β were been reported in patients with Alzheimer's disease (Licastro, et al., 2000). Although assessed in only a small patient cohort, the presence of IL-1β in the cerebrospinal fluid of elderly patients recovering from hip surgery was also found to correlate with the incidence of delirium (Cape, et al., 2014). IL-1β mRNA expression also increased in adipose tissue of aged (23 month) mice, as compared to 2 month old mice (Youm, et al., 2013). Griffin et al (Griffin, et al., 2013) found that IL-1β antagonism, via systemic administration of IL-1RA, improved cognitive function in a LPS-induced mouse model of cognitive decline. Similarly, as compared to aged wild type mice, motor performance and cognitive function was enhanced in aged *Il1r*$^{-/-}$ mice (Youm, et al., 2013). Collectively, these studies support a role for IL-1β in age-associated cognitive impairment. Mechanistically, a recent study in mice has demonstrated that IL-1β and IL-1R expression was increased during ageing, and this was linked to an enhanced capacity to generate pathogenic Th17 cells (Lim, et al., 2014). Whereas IL-1β likely contributes to neuroinflammation and a decline in cognitive function during ageing, recent studies have linked IL-18 to cardiovascular-related pathology. IL-18 was associated with heart failure in a cohort of 2,917 men and women aged between 70-79 years (Driver, et al., 2014). In addition, artery injuries in old rats showed increased fibrinogen deposition that correlated with increased vascular IL-18. The authors found that levels of IL-18 in the injured vasculature were more than 23-fold higher in aged rats than in young rats (Rodriguez-Menocal, et al.,

2014). IL-18 may also contribute to a decline in bone and joint function during ageing. For example, using 10 and 24 week old mice constitutively overproducing IL-18 in the lungs, Takenaka et al (Takenaka, et al., 2014) demonstrated an age-related decrease in body weight and bone mineral density in IL-18 transgenic mice, as compared to wild-type controls. In summary, unhealthy ageing is associated with elevated levels of specific pro-inflammatory mediators such as IL-6, and IL-1β and IL-18 are likely contributors to specific pathological processes during ageing (Michaud, et al., 2013).

2.4 Chronic low-grade inflammation as a driver of unhealthy inflammation

Many mechanisms have been proposed to contribute to unhealthy ageing. These include the effects of diet and postprandial inflammation, a cumulative effect of reactive oxygen species leading to DNA damage, and the role of the growth hormone/insulin-like growth factor axis (Finch, 2010, Junnila, et al., 2013, Wei, 1998). Many of the proposed mechanisms share the concept that a chronic, basal inflammatory state plays a causal role. In 2000, Claudio *Franceschi,* arguing that ageing was the source of a stress response leading to chronic inflammation, coined the term *inflam-ageing* (Franceschi, et al., 2000). His hypothesis proposed two paradoxical characteristics of ageing. On the one hand, immune cells produce less pro-inflammatory cytokines, yet more inflammatory mediators are present in the organism at a basal level. Indeed, this apparent paradox is evident in the literature reviewed in Sections *2.2* and *2.3* above. Inflammatory mediator production by non-immune cells such as adipose tissue could account for such effects (Wu, et al., 2007). Another possibility is that whilst many innate immune responses are impaired during ageing, others are hyper-activated. Below we review the evidence that dysregulated inflammasome activation, through elevated levels of danger signals as well as defects in normal regulatory mechanisms, leads to chronic, low-grade, pathological inflammation during ageing.

3. Inflammasome dysregulation in ageing

3.1 MAMPs and DAMPs in the link between ageing and inflammation

Innate immune cells are finely tuned for sensing changes in the extracellular environment. They do so though cell surface and intracellular receptors that detect cytokines, chemokines, peptide mediators, lipid mediators and metabolites, as well as danger signals in the form of endogenous DAMPs and exogenous MAMPs. Several families of PRRs, some of which can form inflammasomes (see Section *3.2*), detect these MAMPs and DAMPs. An obvious mechanism that could account for increased inflammatory responses at the organismal level during ageing is dysregulated PRR responses via increased levels of MAMPs and/or DAMPs. Age-associated increases in MAMPs can occur through gut leakage. It is now well established that during stress, bacterial products (e.g. endotoxin and peptidoglycan) can be translocated from the intestinal tract to the circulatory system (Gatt, et al., 2007). Indeed, gut leakage occurring during the course of abdominal aortic surgery correlated with an increase in plasma levels of IL-6 and CRP (Kim, et al., 2009). Moreover, ageing has been linked with impaired function of the gut mucosa (Fujihashi, et al., 2000, Meier and Sturm, 2009). For example, a study comparing young and old rats (3 and 28 months) reported that ageing increased gut permeability, as assessed by increased intestinal permeability of the probes mannitol and PEG 400 (Ma, et al., 1992). In addition to differences in the permeability of the intestinal tract, age can potentially impact on the level of response that occurs when bacterial products access the circulation. For example, the elderly displayed a prolonged fever response, enhanced production level of TNF, and a more profound decrease in arterial pressure during endotoxemia (Krabbe, et al., 2001, Krabbe, et al., 2001). Thus, it is reasonable to conclude that elevated levels of MAMPs can contribute to systemic inflammatory effects during ageing.

Host-derived DAMPs provide an additional mechanism of age-related innate immune activation. The literature on specific host-derived DAMPs driving inflammation has been somewhat clouded by the fact that DAMP preparations used to investigate pro-inflammatory effects can sometimes be contaminated with bacterial products, such as endotoxin (Tsan and Gao, 2007). For example, heat-shock protein 60 was originally described as an endogenous host factor that signalled via TLRs (Bulut, et al., 2002, Kol, et al., 1999, Ohashi, et al., 2000), but subsequent studies raised concerns about possible endotoxin contamination of the heat shock protein preparations as the likely mediator of such effects (Bausinger, et al., 2002, Gao and Tsan, 2003). CRP, the concentration of which increases during ageing, was similarly shown to activate endothelial cells (Blann and Lip, 2003, Pasceri, et al., 2000, Venugopal, et al., 2002), but multiple groups raised similar concerns about contaminating endotoxin (Nerurkar, et al., 2005, Taylor, et al., 2005). Such examples highlight possible complications of interpreting data that show pro-inflammatory effects of specific DAMPs, and the need for extreme rigour in sample preparation and analysis, for example the use of the limulus amebocyte lysate assay to assay levels of endotoxin (Levin and Bang, 1968), purification methods that remove contaminating LPS (Ongkudon, et al., 2012), the use of polymyxin B sulphate as an LPS antagonist (Duff and Atkins, 1982, Palmer and Rifkind, 1974), and crude biochemical methods such as protease-treatment and/or boiling. However, even these methods are not fail-safe. For example, low LPS concentrations are also rendered inactive by boiling (Gao, et al., 2006) and non-LPS contaminants such as bacterial lipoproteins are often a major issue. Moreover, when one is considering whether a particular DAMP acts through a PRR already known to directly recognize a specific MAMP, the structural features of that DAMP should be considered. For example, TLR4 detects the lipid A region of LPS. Thus, it is difficult to understand how a protein alone would directly signal via this receptor unless it can initiate receptor dimerization by a distinct mechanism, for example through an allosteric

site. Alternatively, it may be that the protein in question presents lipid A, or a lipid A-like molecule, to TLR4. Indeed, this is a recurring theme in PRR biology. As two examples of this, mouse mammary tumour virus was reported to present LPS for recognition by TLR4 as part of its host evasion strategy (Kane, et al., 2011), and hemozoin produced during *Plasmodium* infections presents parasite DNA for recognition by TLR9 (Parroche, et al., 2007). Despite these significant issues, an abundant literature has described many DAMPs that can directly or indirectly activate specific PRRs.

Well-characterized DAMPs, among many, that can signal through non-inflammasome forming PRRs include oxidised low density lipoproteins and Aβ that signal via TLR4 and TLR6 (Bae, et al., 2009, Miller, et al., 2003, Stewart, et al., 2010), as well as the extracellular matrix protein versican (Kim, et al., 2009) and serum amyloid A (Cheng, et al., 2008, He, et al., 2009) that signal through TLR2. Another extensively studied DAMP is high-mobility group protein B1 (HMGB1), a nuclear protein that binds to nucleosomes and controls DNA bending and gene expression (Kang, et al., 2014). Its release into the extracellular environment, indicative of cell damage or death, can lead to PRR-dependent inflammatory responses. HMGB1 has been reported to signal via multiple PRRs, through either direct recognition or through interaction with specific PAMPs (Bianchi, 2009). Reported signalling mechanisms include (1) forming a complex with dsDNA to signal through TLR9 (Ivanov, et al., 2007, Tian, et al., 2007); (2) binding to the lipid A moiety of LPS, resulting in disaggregation from the cell membrane and transfer to CD14 for signalling through TLR4 (Youn, et al., 2008); (3) directly binding TLR2 to initiate inflammatory responses (Park, et al., 2006, Park, et al., 2004); and (4) interacting with the receptor for advanced glycation end products (RAGE) (LeBlanc, et al., 2014). RAGE is a multi-ligand receptor that was initially identified as a receptor for advanced glycation end products (Bierhaus, et al., 2005, Schmidt,

et al., 1992). RAGE has itself been reported to bind multiple DAMPs apart from HMGB1, for example members of the S100 family of proteins (Hofmann, et al., 1999). Thus, DAMPs and their receptors appear to be particularly promiscuous in the mechanisms by which they drive inflammatory responses, albeit with the caveats regarding MAMP contamination (described above).

3.2 Inflammasome-driven inflammatory responses

In response to certain DAMPs and MAMPs, some PRRs can initiate the formation of an "inflammasome", a cytosolic signalling platform (Latz, et al., 2013, Martinon, et al., 2002). When activated, these signalling hubs trigger the activation of certain inflammatory caspases, most notably caspase-1, through auto-cleavage. Activated caspase-1 in turn cleaves pro-forms of the inflammatory cytokines IL-1β and IL-18 to enable their release into the extracellular environment. Through unknown mechanisms, caspase-1 also triggers an inflammatory form of cell death known as pyroptosis. To date, a sub-set of Nod-like Receptors (NLRP1, NLRP3, NLRC4), the Pyhin family member AIM2 and Pyrin have been shown to be capable of forming functional inflammasomes (Schroder and Tschopp, 2010). Of these, NLRP3 has been the most widely studied. NLRP3 contains a C terminal leucine rich repeat (LRR) domain involved in ligand recognition, a central NACHT domain that defines the family and an N-terminal pyrin domain (PYD) (Sutterwala, et al., 2014) (**Figure 3**). Upon activation, the PYD of NLRP3 recruits the PYD-containing protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) through homotypic interactions. ASC then recruits pro-caspase 1, again through homotypic (CARD-CARD) interactions, to enable auto-activation of this protease and the initiation of downstream signalling.

Whilst the activation of caspase-1 was originally thought to be the hallmark of inflammasome activation, more recent studies have identified a non-canonical, caspase-1-independent inflammasome pathway. In mice, this involves activation of caspase 11 (Kayagaki, et al., 2011). This study found that *Casp1*$^{-/-}$ mice were also deficient in caspase-11, because 129/Sv mice (the background on which the Casp1 knock-out was originally generated) have a natural deficiency in Casp11. Subsequent studies demonstrated that caspase-11 was required for inflammasome activation in response to cytosolic LPS (Hagar, et al., 2013, Kayagaki, et al., 2013, Shi, et al., 2014), and a very recent study has shown that the CARD of caspase-11 directly detects cytosolic LPS (Shi, et al., 2014). Caspase-4 and caspase-5 are the human homologues of mouse caspase-11, and a functional role for caspase-4 in detecting cytosolic LPS in human cells has also been demonstrated (Shi, et al., 2014). Thus, the signalling pathways initiated upon inflammasome activation are not simply a linear series of events downstream of caspase-1.

Inflammasome activation is usually studied in the context of priming and triggering signals. A priming signal, for example through TLR signalling, upregulates the expression of inflammasome signalling components such as Nlrp3 (Bauernfeind, et al., 2009). Priming is also needed for the inducible expression of inflammasome target proteins, most notably pro-IL-1β (Bauernfeind, et al., 2009). However, priming signals can also act independently of transcriptional induction of inflammasome components and target proteins. For example, acute LPS stimulation still primed NLRP3 responses prior to increasing expression of NLRP3 protein (Schroder, et al., 2012). In most cases, the specific mechanisms that mediate inflammasome activation in response to the trigger signal are poorly understood. In the case of NLRP3, this PRR can be activated by many chemically distinct stimuli, acting through diverse mechanisms. For example, ATP-mediated NLRP3 inflammasome activation requires

signalling through the P2X7 receptor (Kahlenberg, et al., 2005), whereas pore-forming toxins such as nigericin act independently of this receptor (Mariathasan, et al., 2006).

The diversity of agents that can trigger activation of NLRP3 suggests that several mechanisms can lead to activation of this PRR or that all signals converge in the generation of a particular cell stress that is sensed by NLRP3. Several mechanisms have been reported, including ROS-mediated Nlrp3 activation (Heid, et al., 2013), potassium efflux-dependent activation of Nlrp3 (Munoz-Planillo, et al., 2013), oxidised mitochondrial DNA-mediated Nlrp3 activation in response to mitochondrial damage (Shimada, et al., 2012), and cathepsin B-mediated NLRP3 activation in response to phagosomal rupture (Dostert, et al., 2009, Hornung, et al., 2008). At a molecular level, the mechanisms by which these pathways could lead to NLRP3 activation are still not well understood, though progress is beginning to be made. For example, ROS-dependent deubiquitination of Nlrp3 has been linked to clustering and activation of this receptor (Juliana, et al., 2012).

4. Age-related factors contributing to Inflammasome dysregulation and Inflam-ageing

4.1 The evidence for a causal role of inflammasome activation in age-related health decline

Since IL1β and IL-18 have been associated with age-related pathologies (Section *2.3*), this raises the possibility that inflammasome activation may have some role in health decline during ageing. Most existing evidence posits NLRP3 as the relevant inflammasome-triggering PRR in this context. Two studies in mouse models are particularly important in this regard. Firstly, Youm et al (Youm, et al., 2012) showed that Nlrp3 plays a causal role in thymic atrophy. Specifically, this study demonstrated that free cholesterol and ceramides accumulated in the thymus of aged mice, and this was associated with inflammasome activation in this organ. Moreover, mice deficient in either *Nlrp3* or *Asc* were protected from

thymic involution, contraction of the T cell receptor repertoire and immune senescence. A subsequent study by the same group (Youm, et al., 2013) showed that 23 month old *Nlrp3*$^{-/-}$ mice had improved glucose tolerance as compared to wild type mice and that age-associated increases in *Il-1β* mRNA expression in adipose tissue and serum IL-18 levels were completely or partially dependent on Nlrp3, respectively. Astrogliosis, a pathological increase in astrocytes that contributes to age-related functional decline (Ransohoff and Brown, 2012), was reduced in *Nlrp3*$^{-/-}$ mice. Microarray profiling of the hippocampal region of *Nlrp3*$^{-/-}$ versus wild type mice also revealed that age-related increases in inflammatory and cell death genes were dependent upon Nlrp3. *Nlrp3*$^{-/-}$ mice were also protected from cognitive decline and bone loss during ageing. These mouse model studies therefore highlight a key contribution of the Nlrp3 inflammasome to age-related features of health decline.

4.2 Inflammasome dysregulation through DAMP accumulation

An obvious mechanism that could contribute to pathological inflammasome activation during ageing is the build up of stimuli that trigger activation of the NLRP3 pathway. Several host products that accumulate during ageing have been characterized as inflammasome activators. These include lipotoxins such as ceramides (Youm, et al., 2012), serum amyloid A (Ather, et al., 2011, Niemi, et al., 2011), cholesterol crystals (Abela, 2010, Duewell, et al., 2010), Aβ (Halle, et al., 2008) and islet amyloid polypeptide (Masters, et al., 2010). For the purposes of this review, we provide a brief overview of just two ageing-associated inflammasome activators, uric acid (UA) and ROS, as specific examples of factors that may contribute to dysregulated inflammasome activation.

UA, the end product of the cellular catabolism of purines, is present in body fluids and at much higher concentrations in the cytosol of healthy cells. When extracellular UA comes into

contact with the high levels of free sodium that are present in the extracellular environment, it is believed to nucleate and form monosodium urate (MSU) crystals (Chen, et al., 2006). MSU crystals were first identified in gout patients in the 18th century (Wollaston, 1797). In more recent times, MSU was shown to trigger the activation of the NLRP3 inflammasome and the production of IL-1β (Martinon, et al., 2006). Indeed, macrophages from mice deficient in inflammasome components such as caspase-1, NLRP3 and ASC, displayed a greatly reduced capacity for MSU-induced IL-1β production (Amaral, et al., 2012). Targeting of the IL-1 axis in gout, with initial clinical trials of the IL-1R antagonist Anakinra, has shown remarkable efficacy (McGonagle, et al., 2008, McGonagle, et al., 2007, So, et al., 2007). Similarly, patients with pseudogout, an inflammatory disease caused by the deposition of calcium pyrophosphate dihydrate crystals (Bennett, et al., 1976), another type of pathogenic microcrystal that activates the NALP3 inflammasome, responded well to IL-1 antagonism (McGonagle, et al., 2008). These findings are of interest in view of the evidence linking MSU accumulation with ageing. For example, a longitudinal study among 80,506 Japanese office workers or their families between 1989-1998 showed that serum UA levels progressively increased with age, independent of changes in alcohol consumption and body mass index over the period of assessment (Kuzuya, et al., 2002). In addition to its causal role in gout, high levels of circulating UA (hyperuricemia) have been associated with various inflammatory diseases, IgA nephropathy (Moriyama, et al., 2014), hypertension (Luo, et al., 2014, Nagahama, et al., 2014) and multiple sclerosis (Moccia, et al., 2014). However, whether the hyperuricemia is a cause or consequence of the disease is still unclear in most of these conditions. Nonetheless, the accumulation of MSU crystals provides one mechanism for inappropriate inflammasome activation during ageing.

ROS have widely been proposed to contribute to the development of many age-related disorders, including atherosclerosis and other cardiovascular diseases (Bonomini, et al., 2008, Griendling and Alexander, 1997), diabetes (Kaneto, et al., 2007), cancer (Benz and Yau, 2008), neurodegenerative disorders (Barnham, et al., 2004), and chronic diseases of the liver (Webb and Twedt, 2008) and lung (Park, et al., 2009). ROS produced by the respiratory chain of mitochondria is primarily generated via complex I (NADH coenzyme Q reductase), though complex III (cytochrome c reductase) can also contribute under certain conditions (Murphy, 2009). Damage by ROS results in lipid peroxidation (Gardner, 1989), protein oxidation (Stadtman, 1990) and mitochondrial DNA mutations (Richter, 1992, Richter, et al., 1988). A possible link between oxidized mitochondrial DNA fragments and ageing was proposed in 1988 (Richter, 1988), and since then, developments in the field have provided further evidence of the role of ROS, as reviewed recently by Zapico and Ubelaker (Zapico and Ubelaker, 2013). In the context of inflammasome activation, ROS appear to be involved in NLRP3 activation in response to multiple stimuli (Cruz, et al., 2007, Dostert, et al., 2008, Jiang, et al., 2012, Joshi, et al., 2014). Baurenfeind et al (Bauernfeind, et al., 2011) showed that diphenyliodonium, an inhibitor of NADPH oxidase-dependent ROS production, impaired LPS-mediated upregulation of *Nlrp3* mRNA in mouse macrophages. In addition, diphenyliodonium only decreased Nlrp3-triggered IL-18 and IL-1β production when cells were exposed to this agent prior to LPS stimulation, and not after. Although this suggests that ROS contributes solely to priming of inflammasome responses, several other studies have provided evidence for causal roles of ROS in triggering inflammasome activation. Zhou et al (Zhou, et al., 2011) showed that mitochondrial ROS can trigger NLRP3 activation, and that the voltage-dependent anion channel was essential for this response. Another report provided evidence that mitochondrial ROS-mediated inflammasome activation involved oxidation of mitochondrial DNA, which was then directly sensed by Nlrp3 (Shimada, et al., 2012).

Another study also reported that mitochondrial ROS mediates cathepsin B release from damaged lysosomes, thus resulting in Nlrp3 activation (Heid, et al., 2013). In summary, evidence for a link between ROS generation and inflammasome priming and/or activation continues to accumulate. Thus, increased or sustained production of ROS could contribute to dysregulated inflammasome activation during ageing and chronic inflammation.

4.3 Inflammasome dysregulation through a failure in control mechanisms

An additional mechanism by which dysregulated inflammasome activation could occur during ageing is through loss of regulatory control mechanisms. For example, type I IFN negatively regulates the Nlrp1 and Nlrp3 inflammasomes (Guarda, et al., 2011). This is of interest given that type I IFN producing pDC are reported to decline in numbers with age (Jing, et al., 2009). Moreover, pDC from aged mice (>18 months), as compared to 2 month old mice, produced less type I IFN in response to TLR9 activation (Stout-Delgado, et al., 2008). This age-associated defect in type I IFN production was also apparent in West Nile virus-infected human DC (73 year old versus 25 year old donors) (Qian, et al., 2011). Hence, impaired type I IFN production during ageing could conceivably lead to defective control of inflammasome activation. This remains to be tested.

Another possible mechanism that could lead to inappropriate inflammasome activation during ageing is impaired autophagy. Autophagy (from the Greek words “auto”: self and “phago”: eating) was first described in 1963 by Nobel-prize laureate Christian de Duve (De Duve and Wattiaux, 1966), although dense intracellular bodies that may have been autophagosomes were observed a few years earlier (Ashford and Porter, 1962, Hruban, et al., 1963). This cellular pathway involves the degradation of cellular components or intracellular microbes enclosed in an autophagosome, a double membrane structure (Deretic, et al., 2013).

This structure then fuses with a lysosome to form an autolysosome, an efficient degradation system. Autophagy is involved in diverse physiological and pathophysiological processes, and thus its dysregulation has been linked to numerous diseases (Mizumura, et al., 2014).

Several studies have implicated autophagy in negative regulation of inflammasome responses (Salminen, et al., 2012). One of the first demonstrations of a link between autophagy and inflammasome activation came from the work of Akira and colleagues, who showed that macrophages from mice lacking an essential autophagy protein Atg16L1 produced elevated levels of IL-1β and IL-18 in response to TLR activation (Saitoh, et al., 2008). More recent studies suggest that autophagy regulates inflammation by destroying ubiquitinated inflammasome components, as well as pro-IL-1β, thus preventing inflammasome activation and IL-1β maturation (Harris, et al., 2011, Shi, et al., 2012). Inflammasome activation and metabolism are intrinsically linked, in particular with respect to mitochondrial activity (Green, et al., 2011). Zhou et al (Zhou, et al., 2011) showed that blockade of autophagy leads to an accumulation of damaged mitochondria within the cell, thus resulting in ROS-dependent NLRP3 activation. In view of this literature, the strong link between ageing and autophagy is of particular interest. Inhibition of autophagy reduced the lifespan of many organisms such as *C. elegans (Melendez, et al., 2003, Toth, et al., 2008)*, *Drosophila melanogaster* (Juhasz, et al., 2007, Simonsen, et al., 2008) and even mice (Harrison, et al., 2009). In addition, there is also direct evidence that autophagy function decreases with age. Cuervo and Dice (Cuervo and Dice, 2000) showed that chaperone-mediated autophagy declines with age, mainly due to decreased levels of lysosome-associated membrane protein type 2a. Additionally, a genome-wide study revealed that autophagy-essential genes such as type III phosphatidylinositide 3-kinases are down-regulated during ageing (Lipinski, et al., 2010), and a genetic screen in *Saccharomyces cerevisiae* also identified 10 autophagy-related

genes associated with an ageing defect in yeast (Matecic, et al., 2010). Collectively, these studies suggest that boosting autophagy function during ageing could potentially limit inflammasome responses and associated pathology, and increase lifespan. Indeed, long-term treatment with rapamycin, which activates autophagy (Kamada, et al., 2000), improved cognitive functions in old mice and this was associated with lower levels of IL-1β in the brain (Majumder, et al., 2012).

5. Conclusion

A recent WHO report indicated that, between 2000 and 2050, the number of over 65 year old adults is expected to double from 11% to 22%, reaching an absolute number of 2 billion people (WHO, 2012). Thus, new treatment modalities for diseases that are prevalent in the elderly are essential. It has long been appreciated that ageing is associated with dysregulation of certain innate immune functions and elevated levels of specific inflammatory mediators. However, evidence for causal roles of the inflammasome pathway in age-related health decline has only recently emerged through studies in mouse models. Most evidence points to the involvement of the Nlrp3 inflammasome, yet at least some of the increase in serum IL-18 levels in aged mice was ASC-dependent but Nlrp3-independent (Youm, et al., 2013). Moreover, the same study showed that some age-related functional declines were Nlrp3-dependent, but IL-1-independent. There are thus likely to be contributions from other inflammasomes and downstream mediators, beyond the NLRP3-IL-1β axis, in at least some aspects of pathological ageing. Nonetheless, direct or indirect targeting of NLRP3-dependent inflammasome responses may ultimately provide opportunities for the treatment and/or prevention of age-related diseases. Long term treatment regimes in animal models using pharmacological inhibitors of NLRP3, IL-1 and/or IL-18 antagonists, and/or novel autophagy activators are now required to determine efficacy, feasibility and possible complications of

such approaches. With respect to complications, host genetics and host microbiomes are likely to be key factors that will greatly influence how successful these approaches will be in individual people. These are important variables that will necessitate careful consideration in the future. Ultimately, time will tell if the ticking inflammasome time bomb can be deactivated for therapeutic benefit during ageing.

Conflict of Interest

The authors declare that there is no conflict of interests regarding this article.

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FIGURE LEGENDS

Figure 1. Contribution of inflammasome dysregulation to *inflam-ageing*. Proposed model linking perturbed innate immune functions, DAMP accumulation and inflammasome dysregulation with age-associated health decline.

Figure 2. Effect of ageing on blood cell populations. During ageing, numbers of different leukocyte populations do not dramatically change, with the exception of NK cells that increase in number and lymphocyte numbers that decrease. In contrast, many innate immune cellular functions such as pro-inflammatory cytokine production, phagocytosis and chemotaxis decline during ageing.

Figure 3. The NLRP3 inflammasome is triggered by age-associated DAMPs. Age-related DAMPs activate the NLRP3 inflammasome, resulting in pyroptosis and the release of the pro-inflammatory cytokines IL-1β and IL-18. This in turn results in pathological low-grade inflammation.