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# Age-dependent dysregulation of innate immunity

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## Preface

As we age, the innate immune system becomes dysregulated and is characterized by persistent inflammatory responses that involve multiple immune and non-immune cell types, and that vary depending on the cell activation state and tissue context. This ageing-associated basal inflammation, particularly in humans, is thought to be induced by factors including the reactivation of latent viral infections and the release of endogenous damage-associated ligands of pattern recognition receptors (PRRs). Innate immune cell functions, such as cell migration and PRR signalling, that are required to respond to pathogens or vaccines are also impaired in aged individuals. This immune dysregulation may affect conditions associated with chronic inflammation, such as atherosclerosis and Alzheimer's disease.

The United Nations projects that the global human population over the age of 60 will increase by more than threefold (to nearly two billion individuals) during the first half of the 21st century, and by 2050 will exceed the size of the global population of young individuals (less than 15 years of age) [1]. This unprecedented growth in the aged population is observed in both developed and developing nations. In the United States, individuals over the age of 65 currently comprise approximately 12% of the population, but account for over 35% of visits to general internists, 34% of prescription drug use, 50% of hospital stays, and 90% of nursing home residents [2] —reflecting in part increased morbidity and mortality from infectious diseases and poor responses to vaccination [3].

With regard to the adaptive immune system, there is evidence for broad-ranging, age-associated alterations in the development and function of B and T cells [4, 5, 6, 7, 8]. The impact of ageing on the innate immune system had been less well studied, until recently. The diverse cell lineages that mediate innate immunity show heterogeneous ageing phenotypes that reflect their developmental, tissue and activation context. Overall, studies in aged mice (generally older than 20 months) and in humans over the age of 65 have shown that activation of the aged innate immune system results in dysregulated inflammation. This dysregulation involves both elevated basal inflammation (especially in humans) and an

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associated impairment in mounting efficient innate and adaptive immune responses to newly encountered pathogens or vaccine antigens. Indeed, a body of evidence, mainly from human studies, indicates that older adults have elevated levels of pro-inflammatory cytokines, clotting factors and acute phase reactants in the steady state [9-11], and the term 'inflamm-ageing' was coined to describe this phenomenon [12]. Evaluation of several[13, 14] (but not all [15]) human cohorts have shown an association between elevated levels of cytokines, particularly of interleukin-6 (IL-6), and decline in innate immune function in older individuals.

The mechanisms that underlie this heightened ageing-associated basal inflammation remain incompletely understood, but seem to involve changes in the numbers and functions of innate immune cells, altered expression of pattern recognition receptors (PRRs), activation of PRRs by endogenous ligands associated with cellular damage, and aberrant signalling events downstream of PRR activation that lead to cytokine secretion. Here, we review evidence, predominantly from mouse and human studies, on ageing-associated alterations in innate immunity; although there are substantial similarities between aged humans and mice, there are also important differences (Table 1). These differences probably reflect various factors, including the characteristics of genetically homogeneous inbred mouse strains, as well as intrinsic species-specific differences between humans and mice[16]. We also discuss the implications of age-associated changes in innate immunity for improving responses to infection or vaccination and for age-associated chronic inflammatory diseases.

## The ageing innate immune cell compartment

Studies in mice have shown that aged haematopoietic stem cells (HSCs) have reduced regenerative potential and fail to efficiently reconstitute myeloablated recipients following transplantation. Moreover, aged mouse HSCs are biased towards myeloid differentiation at the expense of lymphopoiesis [17-20], and there is also evidence for a similar skewing in human HSCs from older donors [21]. The underlying mechanisms probably include both ageing-associated cell-intrinsic and microenvironmental changes[22]. For example, the presence of low levels of lipopolysaccharide (LPS) or chemokines, such as CC-chemokine ligand 5 (CCL5), may compromise HSC regenerative capacity or promote myeloid skewing[23, 24]. In addition, DNA damage in the form of double-stranded DNA breaks seems to be increased in HSCs from older humans [25]. These alterations are associated with a decline in adaptive immunity and may contribute to an increased incidence of myeloid malignancies associated with ageing [26].

Most innate immune cell populations seem to either remain stable in size or decrease with age. For example, studies using the SENIEUR protocol (which selects for successfully aged adults without comorbid medical conditions) have reported no change or a mild decrease in the numbers of neutrophils [27, 28]. In this regard, it is notable that neutrophilia was a risk factor for death in a multivariate analysis of one cohort of aged adults [29]. Monocyte numbers are grossly unchanged in older adults [30, 31], although age-associated increases in $CD14^{+}CD16^{+}$ inflammatory monocytes have been reported [31-33]. Finally, decreased percentages and numbers of plasmacytoid dendritic cells (pDCs) have been reported in older adults[34, 35] (although not in all studies [36]). Likewise, myeloid DCs (mDCs) also appear to be

decreased or unchanged with age[35, 37]. The existing evidence indicates that basal inflammation in older individuals is not associated with increased numbers of myeloid cells, despite the skewing of aged HSCs to the myeloid cell lineage. This reflects the impaired bone marrow homing, proliferative responses and self-renewal capacity seen in aged HSCs with myeloid skewing [20].

## Ageing and innate immune cell migration

Neutrophils are the first cells to migrate to pathogen-infected sites, and their migratory capacity has been extensively studied in the context of ageing. The speed of neutrophil movement, also known as chemokinesis, was found to be unperturbed in older individuals [38], but neutrophils from aged mice show impaired chemokinesis in response to IL-8 [39]. By contrast, chemotaxis (directional movement in response to a gradient of a stimulus) of neutrophils from older adults [38, 40] and mice [41-43] seems to be impaired. Moreover, impaired neutrophil chemotaxis was found to contribute to delayed wound healing in aged mice, a defect associated with lowered expression of intracellular adhesion molecule 1 (ICAM1)[44] (Figure 1). However, diminished chemotaxis can result not only in reduced neutrophil migration to the sites of inflammation but also in defective neutrophil egress from inflamed tissues. In this context, local neutrophil-mediated inflammation was increased in aged, compared to young mice following burn-associated lung injury, and bacterial or viral infection [39, 45, 46]. In the case of burn-associated injury, an age-associated decrease in the expression of CXC-chemokine receptor 2 (CXCR2) by neutrophils and an increase in the expression of ICAM1 by pulmonary endothelial cells contributed to increased neutrophil inflammation in the lungs[39] (Figure 1). The findings that ICAM1 expression is increased in the burn model but is decreased in the wound healing model may reflect tissue context and differences between a systemic (burn) versus local response.

Neutrophil migration *in vitro* can also be facilitated or inhibited depending on the presence of pro-inflammatory stimuli, including IL-8 and tumour necrosis factor (TNF)[47], and such age-associated microenvironmental and tissue-related factors likely contribute to dysregulation of neutrophil activation and migration. Taken together, these results suggest that aged neutrophils have an impaired ability to traffic into and out of sites of infection. Notably, defects in the migration of mouse pulmonary DCs[48] and human monocyte-derived DCs[36] have also been reported, as further discussed below. Such alterations in cell trafficking with ageing could affect the initiation of innate immune responses at sites of infection.

## Ageing and innate immune cell effector functions

### Neutrophils

Neutrophils from older, compared to young, adults showed impaired phagocytosis of opsonized *Escherichia coli* or *Streptococcus pneumoniae* [49-51] and a diminished capacity to kill phagocytosed microorganisms [50, 52, 53]. One potential mechanism contributing to an age-associated defect in intracellular bacteria killing in human neutrophils could involve the diminished expression of dihydroepiandrosterone sulfate (DHEAS), a circulating steroid that promotes superoxide generation in neutrophils [54].

Age-associated defects in neutrophil effector function have been associated with impaired signal transduction functions. Examples include impaired anti-apoptotic responses to granulocyte-macrophage colony-stimulating factor (GM-CSF) mediated through JAK (Janus kinase)–STAT (signal transducer and activator of transcription) tyrosine kinase [55] and phosphoinositide 3-kinase (PI3K)–AKT pathways [56]. Neutrophils from older adults compared to young individuals show reduced basal expression of suppressor of cytokine signalling 1 (SOCS1) and SOCS3, which negatively regulate JAK–STAT signalling [56], and an impaired response to triggering receptor expressed on myeloid cells 1 (TREM1) [57], which is an immunoglobulin superfamily activating receptor that mediates the production of cytokines, chemokines and reactive oxygen species (ROS). This dysregulated signal transduction may reflect alterations in membrane lipid composition and lipid rafts that result in inappropriate localization or retention in membrane signalling domains. For example, the negative regulator SRC homology domain-containing protein tyrosine phosphatase 1 (SHP1) is excluded from lipid rafts following GM-CSF stimulation in neutrophils from young individuals, but is retained in rafts in neutrophils from older individuals [58]. Taken together, these findings indicate that ageing impairs several signalling pathways in neutrophils, including the generation of the respiratory burst and apoptotic pathways, which may result in reduced protection to microbial infection.

Although in early studies neutrophil function was reported to be largely preserved in aged mice [59, 60], a recent study in aged mice indicated that neutrophils infected with methicillin-resistant *Staphylococcus aureus* (MRSA) exhibited decreased production of CXCL1 and CXCL2[53]. Neutrophils also produce neutrophil extracellular traps (NETs), which are scaffolds of extruded chromatin that contain antimicrobial peptides and proteases such as elastase and myeloperoxidase that facilitate the capture and killing of pathogens [61]. NET formation was diminished with age in a mouse model of MRSA infection [53], but it remains to be determined whether NETs are altered in aged humans.

## NK and NKT cells

Natural killer (NK) cells show decreased cytotoxicity, lung infiltration and interferon-γ (IFNγ) production in a model of influenza virus infection in aged, compared to young, mice [22, 62] (Figure 1). In an ectromelia poxvirus model, NK cells from older mice (aged 14-18 months) showed a cell-intrinsic defect in migration to regional lymph nodes [63]. Cytotoxicity of NK cells induced by type I IFNs appears decreased in aged mice [64], as does induced production of IFNγ and granzyme B for specific cytokine combinations [65].

In humans, NK cells can be broadly divided into a $CD56^{low}$ population that has cytotoxic activity and a $CD56^{hi}$ population that is responsible for cytokine production [66]. $CD56^{hi}$ NK cells appear diminished in proportion and cytokine/chemokine secretion with ageing. In older adults, cytotoxicity is decreased on a per cell basis, with an expansion of the $CD56^{low}$ cytotoxic NK cell compartment [67-72]. Notably, this diminished cytotoxicity is associated with an age-associated defect in the mobilization of perforin to the immunological synapse [73]. Elucidating the mechanisms underlying age-associated changes in NK cell function may have particular clinical impact, as impaired NK cell function is associated with increased infection rates and mortality in older adult nursing home residents[74].

Invariant NKT (iNKT) cells are characterized by CD1d-restricted recognition of endogenous and bacterial glycolipids [75]. iNKT cell numbers are increased in aged versus young mice [76]; by contrast, human iNKT cells in the blood decrease with age and show diminished proliferation in response to the CD1d ligand α-galactosylceramide [77, 78]. In mice, NKT cells produce cytokines such as IL-17A, which contributes to immune pathology after viral infection [46]. Consistent with this, studies in non-infectious murine models indicate that ageing enhances inflammatory responses by NKT cells, and that this leads to liver immunopathology[79, 80] (Figure 1). Thus, NKT cells also contribute to innate immune dysregulation with ageing.

### Macrophages and DCs

Various macrophage and DC effector functions in ageing individuals have been studied. Several reports have demonstrated an age-associated decline in nitric oxide production by mouse and rat macrophages [81-82]. However, evaluation of the effects of aging on phagocytosis has produced mixed results. While several studies report preserved phagocytosis of bacterial targets by aged mouse macrophages [83, 84], studies of human monocytes and monocyte-derived DCs (MDDCs) suggest an age-associated impairment in phagocytosis [32, 36], and phagocytosis of apoptotic cells by both mouse macrophages and human MDDCs has been reported to be reduced[36, 85]. These findings raise the possibility of differential age-associated effects on specific phagocytic functions.

Studies of antigen-presenting cells (APCs) generally show impaired function with ageing [86, 87, 88] (with one exception[89]). For example, bone marrow-derived DCs (BMDCs) in aged compared to young mice were defective in controlling tumour growth in a B16 melanoma model [90]. A recent study of infection with a modified version of *Listeria monocytogenes* demonstrated decreased expression of costimulatory proteins and impaired bacterial uptake by $CD8\alpha^+$ DCs in aged mice, particularly at early stages of infection (although LPS-induced costimulatory protein expression was comparable to young mice) [91]. In oral infection of mice with the intracellular parasite *Encephalitozoon cuniculi*, $CD11c^+$ DCs from lymph nodes in adult (not aged) mice had a decreased ability to prime T cells compared to DCs from young mice [92]. This impaired function was associated with an age-associated decrease in IL-12 production by DCs that could be complemented by IL-15 treatment. Microenvironmental alterations, such as disruption of the architecture of the splenic marginal zone, may also contribute to impairments in antigen presentation[93]. Whether these processes are altered in human cells remains unclear; studies to date using relatively small numbers of subjects have shown preserved antigen presentation function in DCs and monocytes [94, 95].

## Ageing and PRR signal transduction

### Alterations in TLR expression

Ageing is associated with impaired PRR signalling which may, in part, be accounted for by the reported alterations in TLR protein expression in innate immune cells from older compared to young individuals. Decreased surface expression of TLR1 has been associated with diminished TLR1/TLR2-induced cytokine production in human monocytes [33, 96]. Age-

associated decreases in TLR1, TLR3 and TLR8 protein expression by mDCs [97] and in TLR7 and TLR9 expression by pDCs [37] have been reported as well. While substantial age-associated decreases in *TLR* gene expression have been reported in mice [98], this is less clear in humans. Concordant changes in both protein and gene expression have been reported, such as the age-associated increase in TLR5 mRNA and protein in adherent human monocytes [99], but in other cases changes in gene expression may not be sufficient to account for changes in TLR protein levels [97]. This suggests that post-transcriptional mechanisms contribute to the decreased TLR protein levels; in human monocytes, total TLR1 protein expression was comparable in cells from young and older adults; thus, the reported decrease in cell surface TLR1 could result from mechanisms involving TLR1 transport to the plasma membrane [96].

## TLR signalling and cytokine production in aged monocytes and macrophages

TLR signalling has a crucial role in linking innate and adaptive immune responses through the induction of expression of costimulatory molecules and pro-inflammatory cytokines. One of the first studies to evaluate TLR function in aged C57BL/6 mice revealed a generalized decrease in *Tlr1-Tlr9* gene expression and TLR-induced TNF and IL-6 production by splenic and peritoneal macrophages, with decreased expression of TLR4 protein[100]. By contrast, in aged BALB/c mice, surface expression of TLR2 and TLR4 on peritoneal macrophages was unchanged. Nonetheless, TLR2- and TLR4-induced TNF and IL-6 production were reduced in macrophages from aged BALB/c mice, and associated with diminished MAPK p38 protein expression[101, 102]. An age-associated impairment in TLR2-dependent cytokine production and signalling was also reported in alveolar macrophages from BALB/c mice, in a model of *S. pneumoniae* infection [103] and in C57BL/6 macrophages responding to *Porphyromonas gingivalis*, with a decrease observed only for IL-6 production in aged BALB/c mice [84, 104]. These findings provide evidence for age-associated decline in mouse TLR function, with variation in the underlying mechanisms that may in part reflect genetic background (Figure 2).

In rhesus macaques, TLR2-TLR6-, TLR4- and TLR9-induced TNF and IL-6 production in monocytes were decreased in aged, compared to young, animals [105]. In humans, monocytes from older adults produce less TNF and IL-6 in response to TLR1/TLR2 stimulation, with an associated decrease in MAP kinase signalling; decreased cell surface expression of TLR1, but not TLR2, was associated with this reduced cytokine production [96]. In studies of cell subpopulations, TLR1/TLR2-induced cytokine production was decreased in all monocyte subsets, including classical $CD14^{hi}CD16^{-}$, inflammatory $CD14^{hi}CD16^{+}$ and non-classical $CD14^{low}CD16^{hi}$ monocytes, in older compared to young adults [33].

TLR-dependent expression of the costimulatory molecules CD80 and CD86 following TLR engagement *in vitro* was altered in monocytes from older adults, thereby potentially affecting the efficiency of antigen presentation. Notably, the extent of TLR-induced CD80 and CD86 expression strongly correlated with antibody response to the trivalent inactivated influenza vaccine [106]. These studies of induced TLR function were notable for the use of multivariate statistical modeling to adjust for potential co-variates such as gender, race,

comorbidities and medication use — such modeling is valuable in human studies given the considerable heterogeneity in human cohorts.

So, monocyte and macrophage function seems to be diminished overall in aged mice and humans compared to young individuals. However, these impairments occur in the presence of dysregulated or inappropriately persistent inflammatory responses. For example, basal and LPS-induced TNF production by $CD14^+CD16^+$ human monocytes are increased with age [32]. In addition, human (adherent) monocytes exhibit an age-associated increase in TLR5-induced production of IL-8 and IL-6 and increases in TLR5-induced p38 and ERK phosphorylation [99]. This increase in TLR5 signalling in monocytes from older adults was not appreciated in non-adherent cells [96], suggesting that adherence to plastic could result in partial activation that uncovered an age-associated pro-inflammatory bias. The maintenance, or even enhancement, of TLR5 activity with ageing might also be potentiated by an inflammatory environment in the setting of infection. Further studies are needed to clarify the underlying mechanisms in view of the potential use of TLR5 agonists as vaccine adjuvants [107] (Box 1), and association of *TLR5* gene expression with human influenza virus vaccine antibody responses in young adults [108]. Another example of dysregulated TLR responses came from studies of *in vitro* West Nile virus (WNV) infection: in WNV-infected macrophages from older adults, STAT1-dependent downregulation of TLR3 expression was impaired, which resulted in an inappropriate persistence of TLR3 activation that might contribute to the increased morbidity and mortality associated with WNV infection in older adults [109].

Finally, the ageing-associated *in vivo* microenvironment is also likely to contribute to functional defects. For example, in a study of human delayed-type hypersensitivity responses to tuberculin purified protein derivative, impaired T cell migration in the skin of older adults was linked to impaired TNF production by skin macrophages and consequent impairment in endothelial cell activation (Figure 1). Notably, skin macrophages from older and young humans produced comparable levels of TNF *ex vivo*, which suggests that factors in the microenvironment are responsible for the impaired function *in vivo* [110].

### TLR signalling and cytokine production in aged DCs

In mice, there is evidence for preserved DC function with ageing; mouse BMDCs[111] and $CD11c^+$ DCs that were isolated from the spleen and lymph nodes showed unperturbed TLR function [89], although lower levels of LPS-induced IL-6 and TNF production by DCs have been reported in a T cell receptor transgenic system [86]. However, an age-associated decrease in type I IFN production by pDCs was found following *in vitro* or *in vivo* challenge with type 2 herpes simplex virus. This ageing-associated dysfunction was associated with decreased nuclear translocation of IFN regulatory factor 7 (IRF7). In addition, TLR9-dependent type I IFN responses in pDCs were augmented by treatment with anti-oxidants and in mice subjected to caloric restriction [112].

Another age-associated alteration of TLR function includes increased IL-23 expression by mouse BMDCs following combined stimulation with the TLR4 ligand LPS and the TLR7 agonist R848; this increase is associated with alterations in histone modification at the IL-23 p19 promoter [113] and similarly resulted in an age-associated increase in the production of

prostaglandin E2 (PGE2) by BMDCs. Notably, PGE2 was found to substantially augment IL-23 production by DCs from aged but not young mice [114]. As IL-23 promotes the differentiation of T helper 17 ($T_H17$) cells, such increases in PGE2 and IL-23 production might underlie the potential increased predisposition to $T_H17$ cell responses reported in aged individuals [115, 116]. An age-associated increase in PGD2 production has been observed in lung tissue of mice infected with respiratory virus, and this increase was associated with impaired migration of pulmonary DCs to regional lymph nodes (Figure 1). Treatment of these mice with PGD2 antagonists resulted in improved DC migration and T cell responses [48].

Primary DCs from ageing humans have been reported to have impaired functions overall.. For example, the production of IL-12 downstream of TLR4 stimulation with LPS was reduced in myeloid DCs from older compared to young individuals [117]. Another study revealed a generalized, age-associated decrease in TLR-induced cytokine production by mDCs and pDCs and this strongly correlated with reduced antibody responses to influenza virus immunization [97]. Other studies of human pDCs have also shown age-associated changes in TLR7- and TLR9-induced cytokine production [37], influenza virus-induced type I and type III IFNs [118, 119], and WNV-induced type I IFN production [120]. Taken together with other reports indicating that human pDC numbers in peripheral blood are decreased with age [34, 35], these findings suggest that the antiviral and antitumour functions of pDCs may be particularly affected in older adults. Strikingly, basal levels of intracellular cytokines in primary mDCs and pDCs have been found to be increased with ageing [97] — a finding that is consistent with the age-associated pro-inflammatory environment. Such a background of high cytokine production may reflect a degree of basal TLR activation that cannot be further augmented with additional TLR agonist treatment, and could contribute to failed innate immune responses to pathogens or vaccines (Figure 2).

Monocyte-derived DCs (MDDCs) from older compared to young adults showed increased TNF and IL-6 production following TLR4 or TLR8 stimulation; and TNF and IFNα production were also increased following exposure to self-DNA *in vitro* [36, 121]. By contrast, *in vitro* assays of phagocytosis and a transwell assay of migration demonstrated reduced function by MDDCs from older adults. These phenotypes were associated with impaired PI3K signalling, which has been implicated as both a negative regulator of TLR signalling and a positive regulator of phagocytic function[36]. However, the possibility that the GM-CSF and IL-4 treatment used to obtain MDDCs could attenuate potential age-associated differences should also be considered.

However, ageing is not always associated with increased TLR signalling in human MDDCs. An age-associated decrease in CD80 and CD86 upregulation and in type I IFN production by MDDCs was observed after infection with WNV. This defect was associated with impaired induction of STAT1 and IRF7 expression[120], similar to findings in primary human pDCs [119]. Furthermore, WNV-infected MDDCs from older compared with young adults showed decreased nuclear translocation of the positive regulator IRF1 and increased expression of the TLR inhibitory protein AXL (also known as UFO) [120], which is a member of the TYRO3/AXL/MER (TAM) receptor family. Following activation of the type I IFN receptor (IFNAR)-STAT1 pathway, TAM receptors are expressed and hijack this pathway

to produce the inhibitory proteins SOCS1 and SOCS3[122]; this mechanism provides a link to the STAT1-dependent decrease in type I IFN production that occurs following WNV infection. Taken together, these findings indicate that the effects of ageing on responses by DCs are complex, with evidence for both inappropriately impaired and augmented responses to PRR engagement that reflect cell type, activation state and tissue context (Table1 and Figure 2).

### Signalling downstream of other PRRs

The effects of ageing on cytoplasmic PRRs, such as the NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), remain to be completely elucidated. Notably, a recent study of aged *Nlrp3*$^{-/-}$ mice implicates the NLRP3 inflammasome in age-associated inflammation in adipose tissue and the brain, with aged knockout animals showing improvement in glucose tolerance and tests of memory and learning [123]. By contrast, aged C57BL/6 mice showed reduced production of IL-1β and IL-18 following NLRP3 activation in the context of influenza virus infection [124]. Taken together, these findings are again consistent with failure in innate immune activation in a setting of dysregulated inflammation (Figure 1).

PRR signalling is known to intersect with macroautophagy, which seems to be defective in various cell types from aged organisms [125]; so it is probable that autophagic responses to intracellular pathogens are also impaired in older individuals. At the same time, defects in autophagy may influence PRR signalling via increased generation of ROS from dysfunctional mitochondria that ordinarily would be subject to macroautophagic degradation. As signalling downstream of both NLRP3 and RLR seem to be influenced by impaired autophagy [126-129], and as ROS production also generally increases with age, it is likely that PRR function in older individuals will be influenced by changes in autophagy [130].

## Systemic factors contributing to innate immune ageing

### Hormonal changes

Factors that are extrinsic to the immune system also contribute to the heightened pro-inflammatory environment associated with ageing. For example, both oestrogen and testosterone decrease IL-6 production *in vitro* and *in vivo* [131-134], and age-associated decreases in oestrogen or androgen production are associated with increases in basal pro-inflammatory cytokine levels [135, 136]. Another non-immune contribution to the inflammatory environment is the neuroendocrine axis linking the central nervous system to the periphery via the hypothalamus. Knockdown of the histone deacetylase Sirtuin 1 in hypothalamic neurons induced a pro-inflammatory activated state in peripheral $CD4^+$ T cells [137]. Indeed, recent studies indicate that mammalian lifespan itself may be linked to an NF-κB-dependent inflammatory state in the hypothalamus, which occurs in aged mice and is linked to decreases in gonadotropin releasing hormone[138]. Further studies will be required to determine whether an analogous mechanism occurs in ageing humans.

### Metabolic changes

Adipose tissue has immunological functions in addition to its well-known functions in energy storage and metabolism. Redistribution of adipose tissue and impairments in

metabolic functions such as insulin sensitivity in older individuals is well described [139], and adipose tissue may be a source for age-associated increases in systemic levels of pro-inflammatory cytokines [140-143]. Increased infiltration of macrophages in adipose tissue of aged mice is reminiscent of that seen in the setting of obesity, and supports the notion of obesity as a form of accelerated ageing in adipose tissue [144]. Additional work on the biology of adipose tissue in the context of ageing will be required to obtain mechanistic insights. However, it is notable that saturated fatty acids and ceramides generated in the setting of lipotoxicity can activate the NLRP3 inflammasome [128, 145]. In addition, a recent study identified a decrease in expression of Dicer, a protein that mediates microRNA processing, in adipose tissue from aged mice and human preadipocytes from older adults. Mice with an adipose tissue-specific knockout of Dicer had increased sensitivity to oxidative stress and expression of markers associated with senescence, which implicates miRNA regulation in adipose tissue ageing and its potential contribution to the age-associated pro-inflammatory environment[146] (Figure 1).

## DAMPs

Intriguing links between DNA damage and the activation of inflammatory responses comprise an additional potential contributing factor to the age-associated pro-inflammatory environment. For example, DNA damage from ionizing radiation has been associated with IL-6 production by epithelial cells[147]. Indeed, IL-1β, IL-6 and IL-8 are components of the senescence-associated secretory phenotype (SASP), which is the secretome of cytokines, growth factors, extracellular matrix proteins and proteases that are produced by senescent cells in response to DNA damage and that modify the local microenvironment [148]. The SASP is also regulated by signalling via p38 MAPK [149] and by cell-associated IL-1α signalling [150]. Notably, in a cohort of nonagenarians, levels of endogenous non-cell associated DNA were strongly associated with inflammatory markers such as C-reactive protein and were an independent risk factor for mortality [151]. Such DNA, which could be released from senescent or necrotic cells, represents potential endogenous damage-associated molecular patterns (DAMPs) that activate innate immune responses. Several studies have shown that necrotic cells can activate the NLRP3 inflammasome, likely through the release of cellular ATP[152-154], but it remains possible that additional PRRs, such as those that recognize nucleic acid motifs, could also participate in innate immune activation by endogenous ligands. In view of these findings, chronic infections, such as with cytomegalovirus (CMV) or other herpesviruses, ongoing endogenous DNA release by apoptotic cells or engagement of innate immune PRRs by other self ligands may all contribute to the increased inflammatory state associated with ageing. While chronic viral infections in humans depend on exposure, it remains unclear whether such pro-inflammatory DNA is intrinsic to ageing *per se*, or instead results from the consequences of comorbid disease or alterations in nutritional status in older adults.

## Chronic and latent viral infections

Chronic viral infections, particularly with human CMV, have been linked to an age-associated pro-inflammatory environment[155]. CMV is known for cycles of asymptomatic reactivation throughout life, and such reactivation could contribute to an inflammatory micorenvironment[156, 157]. However, establishing causality, as opposed to associations, in

humans has remained elusive. Although CMV reactivation has been shown to be associated with increased IL-6 and TNF levels and with impaired responses to influenza virus vaccine [158, 159], a recent longitudinal study that compared CMV seronegative older adults, those seroconverting to CMV and those who remained seropositive over a 10-year period failed to demonstrate an effect of CMV status on markers of inflammation [160]. This study was the first to longitudinally evaluate individuals who seroconverted to CMV, and does not implicate CMV as the dominant driver of an age-associated pro-inflammatory environment. However, the number of new CMV seroconverters in this study was relatively small, and it is also conceivable that multiple rounds of CMV reactivation throughout life are needed to establish a systemic increase in inflammation. Further longitudinal studies over CMV are needed to address these issues.

## Ageing and chronic inflammatory disease

The evidence discussed for age-associated innate immune dysregulation, in the setting of hormonal and metabolic aetiologies and endogenous ligands for PRRs, suggests that the resultant pro-inflammatory state will influence the pathogenesis of not only infectious diseases, but also conditions associated with chronic inflammation. Here, we illustrate the interface of innate immune activation with two examples of diseases that have a high prevalence in older adults — atherosclerosis and Alzheimer's disease.

### Atherosclerosis

Clinical and epidemiological studies have shown that ageing contributes to the development of atherosclerosis [161], which is a chronic inflammatory arterial disease characterized by the deposition of lipids within the arterial wall [162]. Recently, TLR and NLRP3 signalling have been implicated in the development of this disease[163, 164]. It is not clear how ageing enhances the development of atherosclerosis, but elevated basal inflammatory responses may contribute to the chronic inflammation in the disease. It is also poorly understood how an atherosclerotic plaque becomes susceptible to rupture, and the exact nature of the complex thrombotic process after rupture, which include components of the immune system, the coagulation system and stromal cells. Vascular smooth muscle cells (VSMCs) that are harvested from the aortas of aged mice were found to have pro-atherogenic features, including increased production of pro-inflammatory cytokines, such as IL-6, and chemokines, such as CCL2 (Figure 1). TLR4 expression was also increased in aged VSMCs, and increased production of IL-6 was dependent on TLR4 and MyD88 [165]. VSMCs from ageing non-human primates also showed higher production of pro-inflammatory cytokines, such as IL-1β, TNF and CCL2[166]. In this study, the increased inflammatory response correlated with increased mitochondrial oxidative stress. Notably, treatment with resveratrol, which is a naturally occurring phenol that has been shown to extend life span in various experimental models, reversed the ageing-associated pro-inflammatory phenotype of VSMCs. However, it remains unclear how the inflammatory phenotype of aged VSMCs contributes to the pathogenesis of atherosclerosis.

### Alzheimer's disease

Dysregulated innate immune responses in the ageing brain may be relevant to various age-associated neurodegenerative conditions such as Alzheimer's disease (Figure 1). PRRs are highly expressed in the brain, with expression of TLR1-TLR9 reported in both mouse and human microglia. Widespread TLR expression is also found in mouse astrocytes and cortical neurons, although in humans only TLR3 expression has been reported [167]. Notably, TLR-induced cytokine production by mouse microglia is increased in aged compared to young mice. In humans, a gene expression microarray study of four regions of the brain in young (20-59 years old) and aged (60-99 years old) individuals, and in patients with Alzheimer's disease (74-95 years old) revealed marked upregulation of TLR- and inflammasome-associated genes in older adults. In addition, a gene expression signature of microglial activation was also observed in older adults[168].

The effect of TLR activation in the context of neurodegeneration has been investigated mainly in mouse models and seems to be complex. The β-amyloid peptide that is found in amyloid plaques that are deposited in the brains of individuals with Alzheimer's disease induces a TLR-dependent innate immune response that may facilitate β-amyloid clearance [169-173]. However, systemic inflammation, for example by administration of LPS, exacerbates β-amyloid plaque formation and cognitive impairment in a transgenic Alzheimer's disease model [174, 175], and an association between systemic inflammation and Alzheimer's disease has been reported in humans [176]. Recent studies have also implicated the NLRP3 inflammasome in Alzheimer's disease pathogenesis [177, 178]. The beneficial and detrimental effects of innate immune engagement in older adults with Alzheimer's disease may reflect the timing, location and extent of inflammation, and remains an area of active investigation.

## Conclusions and future perspectives

Considerable progress has been made in elucidating age-associated alterations in innate immunity and their consequences. For some cell types, information on the effects of ageing is emerging; one example would be myeloid-derived suppressor cells (MDSCs) — a heterogeneous population of immature myeloid cells with potent T cell suppressive function [179]. MDSCs are expanded in malignancies and other inflammatory states, and are also expanded in aged, compared to young mice [180] and humans [181]. These findings are notable given the substantial age-associated increase in human cancers [182]. A recent report in a mouse model of sterile inflammation described a neutrophil population that constitutively produced IFNγ and that conferred T cell resistance to MDSC suppression — this provides a potential mechanism for innate immune dysregulation that could potentiate chronic inflammation[183]. However, it remains to be seen whether this neutrophil population is present in aged humans or mice.

Understanding the biological basis for altered innate immunity and inflammation in ageing is a challenge with substantial clinical impact, as restoration or preservation of physiological function is likely to be of equal or greater importance than extension of lifespan. The study of well-characterized human cohorts with alterations in functional status, such as the

geriatric syndrome of frailty[184], offers the possibility of linking innate immune function and inflammation with biological mechanisms.

Integrative systems biology approaches are also beginning to yield insights into such mechanisms[185]. The use of computational approaches together with model systems will be crucial as our appreciation of the complexity of the immune system and its relationship to the environment increases. For example, the possibility that the gut microbiome may influence vaccine responsiveness [186], probably through engagement of the innate immune system, will require the synthesis of signatures of vaccine responses from gene expression, proteomic and immunological studies with known age-associated alterations in the composition of the human gut microbiome[187, 188]. These offer future prospects to the field of considerable challenge and opportunity.

## Glossary terms

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| **Monocyte-derived DCs (MDDCs)** | MDDCs can be generated *in vitro* from peripheral blood mononuclear cells in the presence of interleukin-4 and granulocyte-macrophage colony stimulating factor. MDDCs resemble myeloid DCs and may model the differentiation of DCs from monocytes that enter sites of inflammation. |
| **Macroautophagy** | An evolutionarily conserved process in which acidic double-membrane vacuoles sequester intracellular contents (such as damaged organelles and macromolecules) and target them for degradation, through fusion to secondary lysosomes. |
| **Inflammasome** | A multiprotein complex that consists of a NOD- and LRR-containing (NLR) protein, an adaptor protein and pro-caspase 1 and that upon assembly facilitates the caspase 1-mediated cleavage and production of mature cytokines such as interleukin-1β and interleukin-18. |
| **Conventional DCs (cDCs)** | Specialized phagocytic antigen-presenting cells that have the classic stellate DC morphology. Mouse cDCs generally express CD11c, but are highly heterogeneous: $CD8\alpha^+$ and $CD4^+$ cDC subsets in secondary lymphoid organs, and $CD8\alpha^+CD103^+$ and $CD4^+CD11b^+$ cDC subsets in the periphery. Human cDCs are generally termed myeloid DCs and are $lineage^-MHC$ class $II^+CD11c^+$. Human equivalents to the mouse $CD8\alpha^+$ and $CD4^+$ cDC subsets can be distinguished by expression of BDCA3 (CD141) and BDCA1 (CD1c), respectively. |
| **NOD-like receptors (NLRs)** | A family of over 20 nucleotide-binding and oligomerization domain (NOD)-like cytoplasmic pattern recognition receptors that sense pathogens, toxins, endogenous danger signals (such as uric acid) and exogenous crystalline substances (such as alum, silica and asbestos) and induce inflammatory responses.. |

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|---|---|
| **Plasmacytoid DC (pDC)** | An immature dendritic cell (DC) with a morphology that resembles that of a plasma cell.. Mouse pDCs express markers including B220, which is usually associated with the B cell lineage. In humans, pDCs express CD123 and BDCA2 (also known as CD303), and are CD11c-negative. These DCs are the main producers of type I interferons in response to viral infection. |
| **RIG-I-like receptors (RLRs)** | A family of cytoplasmic pattern recognition receptors that are related to the RNA helicase retinoic acid inducible gene I (RIG-I) and that recognize viral single and double-stranded RNA and mediate antiviral responses such as type I interferon production. |

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**Box 1**

### TLR agonists as vaccine adjuvants for the elderly

Recently, specific Toll-like receptor (TLR) activators, such as the lipopolysaccharide component lipid A, have been used as a strategy to boost immune protection following vaccination in aged individuals. This may be accomplished by the production of pro-inflammatory cytokines, which can enhance the responses of aged $CD4^+$ T cells[189]. Administration of the TLR5 ligand flagellin has been associated with preserved innate immune responses in aged mice and elevated inflammatory responses by specific human cell types from older individuals[99, 190]. Indeed, a vaccine construct that comprised flagellin linked to peptides from the influenza virus haemagglutinin afforded protection against lung infection with influenza virus in aged mice [190]. However, the protection induced in the aged mice was still inferior to that induced in young mice, which probably reflects defects in the adaptive immune response. A clinical study using a similar vaccine construct found that people over 65 years of age exhibited a robust humoral response without substantial side effects [191].

Several studies have used the TLR9 ligand CpG-containing oligonucleotides in combination with Fms-like tyrosine kinase 3 (FLT3) ligand as an adjuvanted intranasal vaccine against influenza virus and pneumococcus; both vaccines gave similar responses in young and aged mice [192193]. CpG oligonucleotides added to the pneumococcal conjugate vaccine also restored pneumococcal polysaccharide-specific antibody responses in aged mice [194]. A recent human study found that a TLR4 agonist, glucopyranosyl lipid, enhances the ability of myeloid dendritic cells to respond to influenza virus infection *in vitro*[195], and this was mediated by increased myeloid dendritic cell production of granzyme B[195]. Taken together, these studies indicate that TLR engagement has great potential to enhance immune protection in aged individuals, but targeted use of TLR agonists — alone or in combination — will be essential as different TLR activators may have distinct effects on overcoming defects in the adaptive immune system of older individuals.

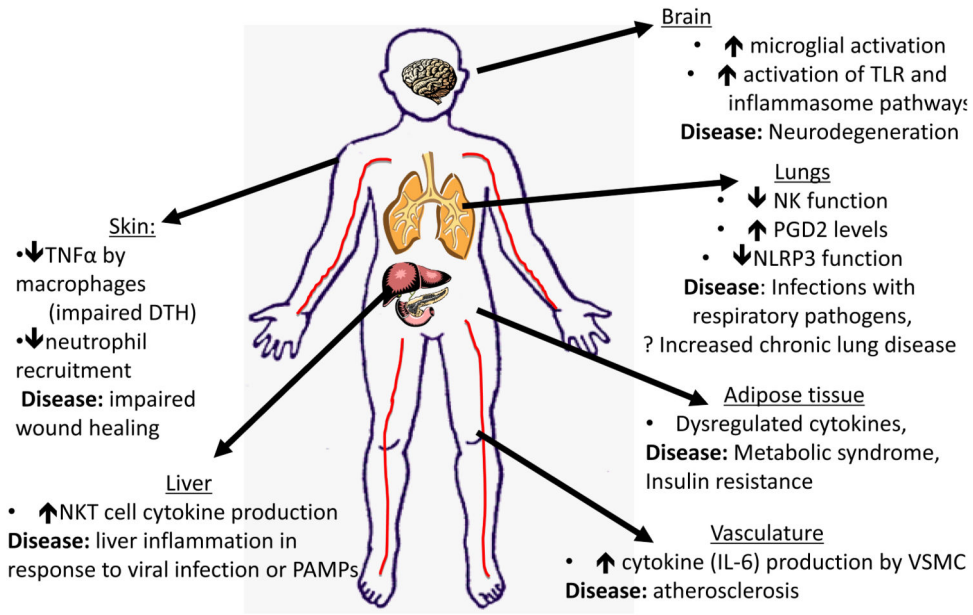


**Figure 1. Organ-specific changes of the innate immune system associated with ageing and disease**

The effects of aging in various organ systems are depicted. Diminished innate immune responses are found in some cases—such as in skin, where a decrease in TNF production by macrophages results in decreased endothelial cell activation and diminished DTH responses. In addition, studies in mice have linked diminished neutrophil recruitment to impaired wound healing. Decreased NK cell function and induced NLRP3 function in response to influenza virus influence infection in the lung. However, there are also examples of dysregulated inflammatory responses found in aging. For example, in the lung following burn injury in a murine model, there is increased neutrophil inflammation. In the liver there is experimental data showing that NKT cells exhibit exaggerated inflammatory responses that contribute to immune pathology during viral infection or to TLR activators. In the brain, TLR and inflammasome signalling are elevated with aging, and are associated with microglial activation. Signalling via the NLRP3 inflammasome contributes to increased age-associated inflammation in adipose tissue as well. Finally, within the vasculature, increased basal IL-6 production by vascular smooth muscle cells has been found in aged rodents and non-human primates and may provide a potential explanation for the increased prevalence of atherosclerosis with aging.

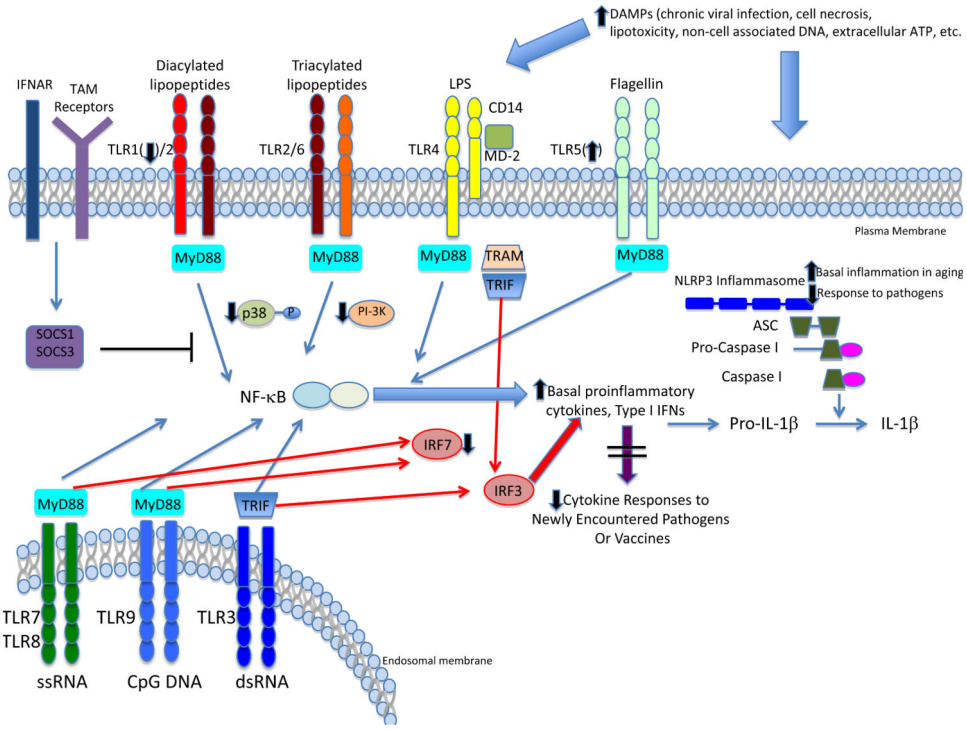


**Figure 2. Effects of ageing on innate immune PRR signalling**

TLR and NLRP3 signalling pathways are depicted, with NF-κB-dependent pathways indicated in blue and type I interferon pathways indicated in red. With aging, elevated levels of PRR ligands (DAMPs) arising from cell damage, necrosis, lipotoxicity or other causes contribute to an elevated pro-inflammatory state, as manifested by increased basal levels of proinflammatory cytokines that may result in part from both TLR and NLRP3 signalling. Such basal activation may restrict responsiveness to new pathogens or vaccines, resulting in innate immune failure and impaired adaptive immune responses. Changes in protein expression for TLR1 and TLR5 in humans are indicated; results in mice suggest either a widespread decrease in TLR gene expression, or preserved expression with changes in intracellular signaling proteins (e.g. p38 MAP kinase). Abbreviations: TLR—Toll-like Receptor; IFNAR—type I Interferon receptor; MyD88—myeloid differentiation primary response 88; TRIF—Toll/IL1 receptor domain-containing adapter-inducing interferon-β; TRAM—TRIF-related adaptor molecule; IRF3—interferon regulatory factor 3; IRF7—interferon regulatory factor 7; NLRP3—NOD-like receptor family, pyrin domain containing 3; ASC—Apoptosis-associated speck-like protein containing a CARD.

| Cell type | Decreased in ageing | Increased in ageing |
|---|---|---|
| ***Human*** | | |
| Haematopoietic stem cells | • Lymphoid differentiation[21] | • DNA damage [25]<br>• Myeloid biased differentiation[21] |
| Neutrophils | • Chemotaxis[38, 40]<br>• Intracellular bacterial killing[50,52,53] (Unchanged in mice [60])<br>• Phagocytosis[49-51]; (Unchanged in mice [60])<br>• GM-CSF and TREM1 signalling[55-57] | |
| Monocytes | • TLR1/2-induced pro-inflammatory cytokine production[33,96]<br>• TLR-induced co-stimulatory molecule expression[106]<br>• TLR1 and TLR4 expression[33,96] | • TLR5 expression[99]<br>• TLR4-induced cytokine production (CD14+ CD16+ monocytes)[32] |
| Macrophages | • WNV-induced DC-SIGN signalling[109]<br>• TNF production (in the skin)[110] | • WNV-induced STAT1 phosphorylation[109]<br>• WNV-induced TLR3 expression[109] |
| Monocyte-derived DCs and myeloid DCs | • TLR-induced cytokine production[97,117,120]<br>• WNV-induced type I IFN production[120]<br>• Phagocytosis and migration[36]<br>• PI-3 kinase activity[36] | • LPS- and ssRNA-induced cytokine production[36,121]<br>• Basal cytokine production[97] |
| Plasmacytoid DCs | • TLR-induced cytokine production[37,97,118,119,120] | • Basal cytokine production[97] |
| ***Mouse*** | | |
| Haematopoietic stem cells | • Lymphoid differentiation[17, 19, 20] | • Cytokine- and chemokine-mediated damage[23,24]<br>• Myeloid biased differentiation[17,19,20] |
| Neutrophils | • Chemotaxis[41-44]<br>• NET formation[53] | • Neutrophil inflammation[39,45,46] |
| Macrophages | • Cytokine production[84,98,101-104]<br>• TLR1-TLR9 expression (strain dependent)[84,98,101,102] | |

| Cell type | Decreased in ageing | Increased in ageing |
|---|---|---|
| | • MAPK signalling[101,102] | |
| Monocyte-derived DCs and myeloid DCs | • Costimulatory molecule expression (CD8α$^+$ subset; unchanged or decreased in CD11c$^+$ BMDCs)[86,89,91,112]<br>• Antigen uptake (CD8α$^+$ subset; unchanged or decreased in CD11c$^+$ BMDCs)[86,87,91]<br>• Induced NLRP3 function (BMDCs)[124] | • PGE2 (BMDCs)[114]<br>• TLR-induced IL-23 (BMDCs)[113]<br>• Basal NLRP3 function[123] |
| Plasmacytoid DCs | • Type I IFN production[112]<br>• IRF7 nuclear translocation[112] | |