Can blocking inflammation enhance immunity during ageing?

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Abbreviations

β-gal - β-galactosidase

CMV - Cytomegalovirus

COX - cyclooxygenase

CRP - C Reactive protein

DAMPs - damage associated molecular patterns

EBV - Epstein Barr Virus

HMGB1 - high mobility group box 1

IL - Interleukin

LPS - Lipopolysaccharide

MAP - mitogen-activated protein

MMP - matrix metalloproteinases

mTOR - The mammalian target of rapamycin

p16 - CDK4/6 inhibitor p16INK4A

PD-1 - Programmed cell death protein -1

PDL-1 - Programmed death ligand - 1

PGE_2 - Prostaglandin E2

PRR - pattern recognition receptor

SASP - senescence associated secretory phenotype

T_{EMRA} - T effector memory cells that re-express CD45

TGFβ - Transforming growth factor

TLR - Toll-like receptor

TNF - Tumour necrosis factor

TORC1 - mTOR complex 1

TORC2 - mTOR complex 2

Abstract

Ageing is a global burden and the increase in lifespan does not increase in parallel with health-span. Therefore, older adults are currently living longer with chronic diseases, increased infections and cancer. A characteristic of ageing is the presence of chronic low grade inflammation that is characterised by elevated concentrations of IL-6, TNFα and C-Reactive protein (CRP) that has been termed inflammageing (1). Previous studies have demonstrated that chronic inflammation interferes with T cell response and macrophage function and is also detrimental for vaccine responses. This raises the question of whether therapeutic strategies that reduce inflammation may be useful for improving immunity in older adults. In this review we discuss the potential causes of inflammageing, the cellular source of the inflammatory mediators and the mechanisms by which inflammation may inhibit immunity. Finally, we describe existing interventions that target inflammation that have been used to enhance immunity during ageing.

1. Introduction:

Ageing results in increased susceptibility to infections, reduced vaccine responses and increased susceptibility to cancers (2-4). This is due to changes in both the adaptive and innate immune system which have been reviewed previously (5, 6). The focus of this review is to describe how inflammation and in particular the phenomenon of inflammageing impact on the immune system and to discuss current therapies which are being developed to counteract the inflammatory processes that occur with ageing.

2. Inflammageing

Inflammageing, a term first proposed by Claudio Franceschi, is the state of chronic low-grade sterile inflammation which is observed with age. It is characterised by high serum concentrations of C Reactive protein (CRP) and other inflammatory mediators such as Interleukin (IL)-6, IL-8 and TNFα (1). The increase in these inflammatory mediators occurs in healthy older adults in the absence of overt inflammatory disease. However, elevated circulating concentrations of IL-6, CRP and Tumour necrosis factor (TNF)α predicts frailty in older subjects (7, 8). Inflammageing is also associated with increased risk of mortality, in healthy and frail older adults (9-12). In addition to elevated IL-6, CRP and TNFα, IL-1β and inflammasome related genes are also good predictors of all-cause mortality (13). Conversely lower levels of inflammatory cytokines in the peripheral blood correlate with good health outcomes and reduced risk of death of older adults (9).

It has also been shown that inflammation and inflammageing associated cytokines are linked with poor cognitive function and elevated concentrations of plasma IL-6 and CRP are associated with cognitive decline (14-16). Furthermore, inflammageing is associated with chronic diseases such as type 2 diabetes, rheumatoid arthritis and even Alzheimer's disease, however whether this is cause or effect requires further investigation (17).

3. Inflammation and immunity

An acute inflammation is necessary to initiate an immune response against an invading pathogen. After the initial inflammatory response there is a period of resolution which occurs to prevent unnecessary tissue damage and to restore tissue homeostasis (18). However, there is accumulating data showing that chronic inflammation can inhibit immunity *in vivo*. As elevated inflammatory responses are detrimental for vaccine efficacy against influenza (19), yellow fever (20) and hepatitis B. In addition, excessive inflammation in particular TNFα production is linked to decreased killing and clearance of *Streptococcus pneumonia* in

macrophages in an aged mouse model of infection (21). This may occur in part by the induction of premature monocyte egress from the bone marrow by TNFα that impairs their function. Interestingly blockade of TNFα restores monocyte function in old animals, showing that the effect of chronic inflammation can be reversed (22).

Elevated inflammation can also inhibit the response to cutaneous recall antigens *in vivo.* Older humans have decreased response to challenge with antigens such as tuberculin PPD, *candida albicans* antigens and varicella zoster virus (VZV) antigens in the skin compared to young individuals (23). However this was not due to a decrease in the number of circulating or resident memory T cells (23, 24). Instead these subjects exhibit elevated inflammatory responses induced by the injection itself, the extent of which was negatively correlated with their ability to respond to the antigen (25). The temporary inhibition of systemic inflammation with an oral p38 mitogen-activated protein (MAP) kinase inhibitor enhances the response to antigen in older subjects indicating the direct association between excessive inflammation and immune inhibition (26).

4. Source of inflammation

The exact source of elevated inflammation during ageing is may be due to a combination of the following mechanisms that are accentuated in older adults. These include chronic viral infection leading to immune activation, increased inflammatory mediator secretion from visceral fat, increased gut permeability resulting in leakage of bacterial components into the circulation, increase in damage associated molecular patterns (DAMPs), altered immune resolution and accumulation of senescent cells, as shown in Figure 1.

4.1 Chronic viral infections

Chronic infections, which cause a lifelong latent infection, are believed to lead to long term activation of the immune system over time, contributing to inflammageing. The most studied example is cytomegalovirus (CMV) infection that induces a lifelong latent infection after the primary infection, and the virus reactivates periodically and initiates a subclinical immune response. A large proportion of T cells in seropositive older subjects are CMV-specific (27, 28) and these cells are highly differentiated and express senescence-associated markers like CD57 and KLRG1. Furthermore these cells produce high levels of inflammatory cytokines such as IL-2, IFNγ and TNFα after activation (29) that may contribute to inflammageing. Individuals who are CMV seropositive and exhibit elevated CRP levels have increased all-cause mortality as compared to CMV seropositive subjects with low CRP levels

(30). However, the impact of CMV infection on the elevated inflammation in older subjects is controversial (31).

4.2 Increased visceral fat

Obesity and in particular accumulation of visceral fat is highly associated with inflammatory cytokine production (32). Visceral fat is an inflammatory site, that has an infiltration of mononuclear phagocytes, B cells and T cells which contribute to the production of inflammatory cytokines such as IL-6, IL-1β and TNFα (33). In young obese individuals, there is an alteration in their circulating leukocyte populations, that resemble the cells found in older adults. There is an increase in circulating end stage senescent-like $CD4^+$ and $CD8^+$ cells that secrete high levels of inflammatory cytokines after activation and decreased naïve T cells (34). Apart from changes in immune cells resident in fat tissue, there is an increase in visceral adiposity during ageing, due in part to the age related decrease in muscle (35). This increase in visceral fat will contribute to inflammageing due to the inflammatory cytokines produced from the adipocytes themselves (32).

4.3 Gut permeability

Studies performed from aged mouse models have shown that older mice have more permeable intestines with a breakdown in cell-to-cell contacts which leads to leakage of gut contents into the blood stream (21, 36). This results in an increase in bacterial components such as Lipopolysaccharide (LPS) in the circulation which activate circulating mononuclear phagocytes through pattern recognition receptor (PRR) expressed by the monocytes and results in production of inflammatory cytokines such as TNFα and IL-6 (21, 22). In addition there are alterations in the microbiome of older adults that renders them distinct from younger cohorts (37). Ageing is associated with an increase in opportunistic pro-inflammatory bacteria, termed ‘pathobionts’, which are normally only observed in low numbers in young guts (38). Older adults with the most evident altered gut microbiome had elevated circulating inflammatory cytokines, implying that inflammageing is linked to alteration in microbiome (39). However, whether this dysbiosis is as a result of altered gut permeability rather than causative of increased inflammation still warrants further investigation. Evidence from an aged drosophila model have shown that microbiome dysbiosis precedes the increased gut permeability observed with age and thus microbiome dysbiosis could be a causative factor in the increase gut permeability seen with age (40).

4.4 Increase in DAMPs

DAMPs are endogenous cellular components which are released at times of injury, stress or cell death. DAMPS can consist of a variety of cellular products including; the S100 family of calcium binding proteins, histones, genomic or mitochondrial DNA or other secreted factors such as ATP, uric acid or heparin sulphate. This is not an exhaustive list and DAMPs have been extensively reviewed previously (41). When DAMPs bind to their PRR receptor there is an increase in inflammatory cytokine production from the cell.

It is proposed that the processes involved in ageing result in increased DAMP production which contribute to inflammageing (42). There is limited human data to support this hypothesis; however in aged murine studies it was observed that there was elevated levels of high mobility group box 1 (HMGB1) has been observed in old mice (43), HMGB1 is an alarmin family member and binds to surface Toll-like receptor (TLR)2 and TLR4 resulting in the production of inflammatory cytokines including IL-6 (44). In addition, NLRP3 inflammasome, an innate immune sensor that is activated in response to an array of DAMPs, was found to be elevated in aged mice. Removing the NLRP3 gene from these mice resulted in a reducing in aged related inflammation; implying DAMPs are involved in the process of inflammageing (45).

4.5 Ineffective immune resolution

After an acute inflammatory response to an infectious agent or traumatic event such as a wound healing response there is a period of immune resolution where the tissue is restored back to its original state (18). Cells involved in resolution of inflammation include mononuclear phagocytes and stromal cells in addition in addition to lipid mediators such as prostaglandins that are involved (18). A recent study has shown that although the onset of acute inflammation between older and younger adults is similar the resolution of the inflammation is impaired in the older adults (46). Indeed there was reduced efferocytosis and clearance of apoptotic neutrophils by the mononuclear phagocytes during the resolution phase of the inflammatory response, which led to a failure to resolve inflammation. This was due in part to reduced expression of TIM-4, a receptor that recognizes apoptotic cells, on mononuclear phagocytes (46). This means that acute inflammatory events are not efficiently resolved in older individuals which could contribute to inflammageing.

4.6 Senescent cell accumulation with age

Cells entering a state of senescence experience irreversible growth arrest that occurs as a result of the irreparable cell damage e.g. DNA damage, telomere erosion or oxidative stress (47). Senescence is a protective process that prevents the proliferation of damaged cells and is viewed as a tumour suppressor mechanism (48, 49). However recent evidence suggests that senescent cells may have beneficial effects and can contribute to wound healing in the skin (50). Senescent cells are characterised by the expression of CDK4/6 cyclin inhibitor p16INK4A (p16) and/or β-galactosidase (β-gal) however this is not an exhaustive list and markers of senescence have been reviewed extensively elsewhere (47).

Ageing is associated with accumulation of senescent cells throughout the body and has been shown to occur in every experimental species and organ studied to date, including mouse and primate models (51-54). In humans senescent cells accumulate in the skin (55, 56) and kidney (57) during ageing. The cell types that are senescent in these tissues include fibroblasts, melanocytes and endothelial cells (50, 56, 58). Senescent cells may accumulate due to long-term exposure to DNA damaging agents such as ultra violet B (UVB) and exposure to pollutants (47). However a recent paper showed that there is reduced elimination of senescent cells during ageing that would also account for their accumulation (59). This reduced clearance may be due in part to the expression of HLA-E by senescent cells that binds to the inhibitory receptor NKG2A expressed on NK and $CD8^+$ T cells which inhibits their cytotoxic activity (56). It is possible that other inhibitory receptor/ligand pairs may also be involved in this evasion strategy that enables senescent cell persistence during ageing.

Senescent cells can secrete a range of inflammatory cytokines (such as IL-1β, IL-6 and TNFα), chemokines (such as CCL2 and IL-8), growth factors (such as fibroblast growth factor) and matrix metalloproteinase (MMP) (such as MMP1 and MMP3). DAMPs such as HMGB1, are also secreted contributing to the inflammatory phenotype of senescent cells (43). This secretion of pro-inflammatory mediators is known as the senescence associated secretory phenotype (SASP). Multiple components of the SASP including CCL2, Transforming growth factor (TGFβ) and IL-1α have the ability to drive senescence in a paracrine manner in nearby non-senescent cells, thus overall increasing the number of senescent cells (60). All components of SASP contribute to the local inflammatory environment and may contribute to the inflammageing phenomenon (61).

Although the majority of senescence research has focussed on cells in tissues, there are also populations of circulating senescent-like leukocytes that accumulate during ageing (62). Examples of senescent-like leukocytes include terminally differentiated $CD4^+$ and $CD8^+$ T effector memory cells that re-express CD45RA (T_{EMRA}), these cells have low proliferative capacity and secrete inflammatory mediators (63-65). Terminally differentiated NK cells also

accumulate during ageing and these $CD16^{dim}KLRG1^{+}$ cells have increased inflammatory cytokine production (66). All these senescent-like leukocytes in older individuals may also contribute to inflammageing.

5. How does inflammation inhibit immunity?

There are direct effects that inflammation has on immunity, such as the suppressive effect of TNFα on T cell receptor signalling (67) and monocyte phagocytosis (22). There are also other mechanisms by which inflammation inhibits immunity, including increasing the expression of inhibitory receptors, increasing the number and function of $Foxp3^{+}$ T regulatory cells (Tregs) and increasing monocyte infiltration of the tissue.

5.1 Increase of inhibitory ligands and receptors

There is increasing evidence that inflammageing associated cytokines can increase expression of inhibitory ligands on immune cells. An example of this is TNFα, which increases expression of Programmed death ligand 1 (PDL-1) on antigen presenting cells such as mononuclear phagocytes (68). PDL-1 binds to Programmed cell death protein-1 (PD-1) that is expressed on T cells, that leads to apoptosis of the cell. The increase in PDL-1 expression is particularly relevant as increased expression of PD-1 on T cells has been shown on in the skin and peripheral blood populations of these cells in older adults that renders them more susceptible to inhibition (24).

5.2 Inflammation effects on $Foxp3^{+}$ Tregs

Tregs are defined by the transcription factor Foxp3, and they play an important role in maintaining immune homeostasis, as in the absence of these cells, there is widespread autoimmune and inflammatory disease which leads to early death (69, 70). There are increased number of $Foxp3^{+}$ Tregs in the skin of older subjects at baseline and in response to antigen that contribute to decreased cutaneous antigen-specific immune responses during ageing (71, 72). It has been proposed that the accumulation of $Foxp3^{+}$ Tregs in the skin of older adults may be due to inflammatory processes since $Foxp3^{+}$ Tregs are recruited to sites of inflammation (73). Interestingly, inflammageing associated cytokines such as TNFα can increase $Foxp3^{+}$ Treg number and induce them to become more suppressive (74). As a result, inflammageing may induce increased numbers and function of $Foxp3^{+}$ Tregs that can inhibit immunity.

5.3 Senescent cells recruit inflammatory cells via SASP

One major component of the SASP is monocyte chemoattractant chemokines such as CCL2 (56). When monocytes are recruited into tissues and exposed to inflammatory signals, they upregulate immune resolution pathways such as CD39/CD73 and PDL-1 which all of which may have a role in inhibiting immunity (75, 76). Indeed in patients with coronary artery disease, their mononuclear phagocytes from the periphery and atherosclerotic plaques have increased expression of PDL-1 which specifically inhibits antigen-specific T cells in a PD-1 dependent manner (75). It has been shown that there is a negative correlation between the number of monocytes recruited to a site of inflammation and cutaneous antigen-specific immunity (26).

5.4 Immunoregulatory SASP components

Not all components of the SASP can be considered directly inflammatory. Indeed, TGFβ an early component of SASP (77), has been shown to have the potential to generate $Foxp3^+$ Tregs from $CD4^+$ T effector cells (78). Another SASP component is the lipid mediator Prostaglandin E2 (PGE_2), which is a downstream of cyclooxygenase (COX)2 (79, 80). PGE_2 can promote a more tolerogenic environment by increasing production of the immunoregulatory cytokine IL-10 from mononuclear phagocytes as well increasing the number and function of $Foxp3^+$ Tregs (81, 82). In addition, PGE_2 has been shown to inhibit the antigen-specific immunity by blocking proliferation of $CD8^+$ T cells in response to viral antigens (83, 84).

6. Therapeutic targets of inflammageing

Due to the substantial clinical data linking inflammageing with reduced immunity, health and increased mortality in older adults, it has become a crucial therapeutic target in older adults. The current therapies for reducing inflammation that have been proposed include the removal of senescent cells and mTOR and p38-MAPK Kinase inhibition (Figure 2).

6.1 Senescent cells

As senescent cells are a major contributor to the inflammageing process they are an exciting target for reducing inflammageing. Mouse models have been developed where senescent cells can be specifically removed *in vivo* and these studies showed that these animals have

increased lifespan, improved fitness and reduced fur loss (85, 86). Indeed, removal of senescent cells even after onset of age-related disorders, such as sarcopenia and cataracts, resulted in an attenuation of disease pathology (87). As a result of these exciting murine studies, therapies to remove senescent with drugs termed senolytics, has been an active area of research. Assessments of senescent cell behaviour *in vitro* - identified that anti-apoptotic/pro-survival pathways such as BCL2, p53 and CDKN1A and also Phosphoinositide 3-Kinase δ signalling pathways may represent specific pathways that can be targeted for their elimination (88, 89).

Senolytics that have been tested in aged mouse models include the combination of dasatinib and quercetin that significantly reduced vascular pathologies (90). ABT263, a specific inhibitor for BCL2 and BCL-x, was utilised in an aged mouse model and has resulted in a rejuvenation of hematopoietic stem cells (91). Inhibitors of heat shock protein (HSP)90 have prevented onset of age-related pathologies in mice (92). All these senolytic agents have been shown to significantly reduce the senescent cells in the mice and while they show great promise in mouse models, they have yet to be translated to humans and this is an area of intense investigation.

Another potential therapeutic area is to unleash the activity of the individuals own immune system against senescent cells. Since there are strategies that enable the evasion of senescent cells from immune surveillance, preventing this inhibitory axis would facilitate the recognition and removal of senescent cells in old subjects. As the expression of the inhibitory receptor HLA-E prevents NK and $CD8^+$ T cells from killing senescent cells (56) the blocking interactions between the negative ligand HLA-E and its receptor NKG2A would be a strategy to enhance senescent cell clearance *in vivo*. An anti-NKG2A monoclonal antibody, Monalizumab, has been developed as a check point inhibitor, that has been shown to enhance anti-tumour immunity via enabling the NK and $CD8^+$ T cell to kill tumours cells that also express HLA-E (93, 94). It is possible that Monalizumab may also have the potential to be used as a senolytic agent to remove senescent cells from older adults and this requires further investigation.

6.2 mTOR

The mammalian target of rapamycin (mTOR), is comprised of two distinct protein complexes mTOR complex 1 (TORC1) and mTOR complex 2 (TORC2) that are involved with numerous cellular processes including inflammation. mTOR is involved in many inflammatory process, in particular mTOR signalling is downstream of a number of innate immune cell receptors such as TLRs including TLR4, cytokine receptors such as IL-15 and lipid receptors such as

Prostaglandin receptors, all of which can increase inflammatory mediator production from cells (95). In addition, mTOR has been shown to be a regulator of the SASP in senescent cells via promoting IL-1α production (96). mTOR inhibition, via Rapamycin, has been shown to increase life expectancy (97). However, more recently it has also been used to improve vaccine responses in older adults *in vivo* (98). Mannick *et al* treated older subjects with a specific TORC1 inhibitor called RAD001 prior to Influenza vaccination, they found that there was an enhanced response to vaccination as determined by circulating antibody titres. This improvement in vaccine response was proposed to be due to reduced expression of the inhibitory receptor PD-1 on circulating $CD4^+$ and $CD8^+$ T cells (98). A subsequent study by the same group, demonstrated that TORC1 inhibitor treatment prior to vaccination also significantly reduced influenza infections in older subjects (99). However, it is not clear if Rapamycin is acting directly on the inflammation in these subjects on some other process to enhance vaccine efficacy.

6.3 p38 MAP Kinase

p38 MAP Kinase has been shown to be a major signalling molecule upstream of SASP production from senescent fibroblast and from $CD8^+$ T_{EMRA} cells (100-102). It was shown that a non-specific inflammatory response occurs after mild tissue injury after saline injection in the skin of older but not adults and that this was associated with to p38 MAP Kinase signalling (26). The older subjects also has increased numbers of senescent cells in the skin compared to younger individuals. The observed inflammatory response was reminiscent of inflammageing and correlated negatively with their response to recall antigen challenge (varicella zoster virus antigens) in the skin (26). To test directly if the inflammation observed was responsible for decreasing the immune response, old subjects were pre-treated with an oral p38 MAP Kinase inhibitor (Losmapimod) for four days before injection of the antigen (103). It was found that blocking p38 *in vivo* significantly increased cutaneous immunity that was associated with an increase in T cell recruitment to the site of antigen challenge (26). Therefore, in addition to senescent cell elimination as a strategy to reduce inflammation, the inflammatory response itself can be manipulated in older individuals with benefit to immunity. It remains to be determined if the inhibition of inflammation may also alleviate other facets of frailty during ageing. However, while short term inhibition would be acceptable, longer term inhibition, especially with p38 MAP Kinase inhibitors is associated with hepatotoxicity (104).

7. Future perspectives

Inflammageing is caused by a combination of age-related defects including increased DAMP production, increased gut permeability, increased visceral fat, chronic infections and increase in senescent cell numbers. Senescent cells contribute to inflammageing due to their SASP production which includes a wide range of inflammatory cytokines and DAMPS. Therefore, strategies to remove the senescent cells from the body are a promising therapeutic target. There has been extensive research in mouse models to show that removal of senescent cells from an old mouse renders the mouse young again. However, what the long-term implications are for removing senescent structural cells, such as fibroblasts, from tissues when they make up a major proportion of the tissue structure needs further investigation. An exciting potential drug candidate to targeting inflammageing is Metformin - which activates the AMP-activated protein kinase signalling pathway and thus blocking inflammatory-cytokine signalling, has been successful used as a long-term therapy in older adults as a first-line therapy for type 2 diabetes, it has been shown to improve cardiovascular health in these individuals (105). However, strategies that target inflammatory signalling pathways using Rapamycin and Metformin have been utilised with some success, but the effect of longer-term inhibition and potential side-effects are not clear at present.

Current therapies that have been developed utilise a short-term inhibition of inflammation to boost immunity without side effects in older individuals may be of benefit to as an adjunct to vaccination and/or anti-tumour therapy. A combination of approaches including one or more of senolytic drug, checkpoint inhibitors (anti-NKG2A) and anti-inflammatory agents may be required for optimal blocking of inflammageing to reduce frailty and enhance immunity in older adults.

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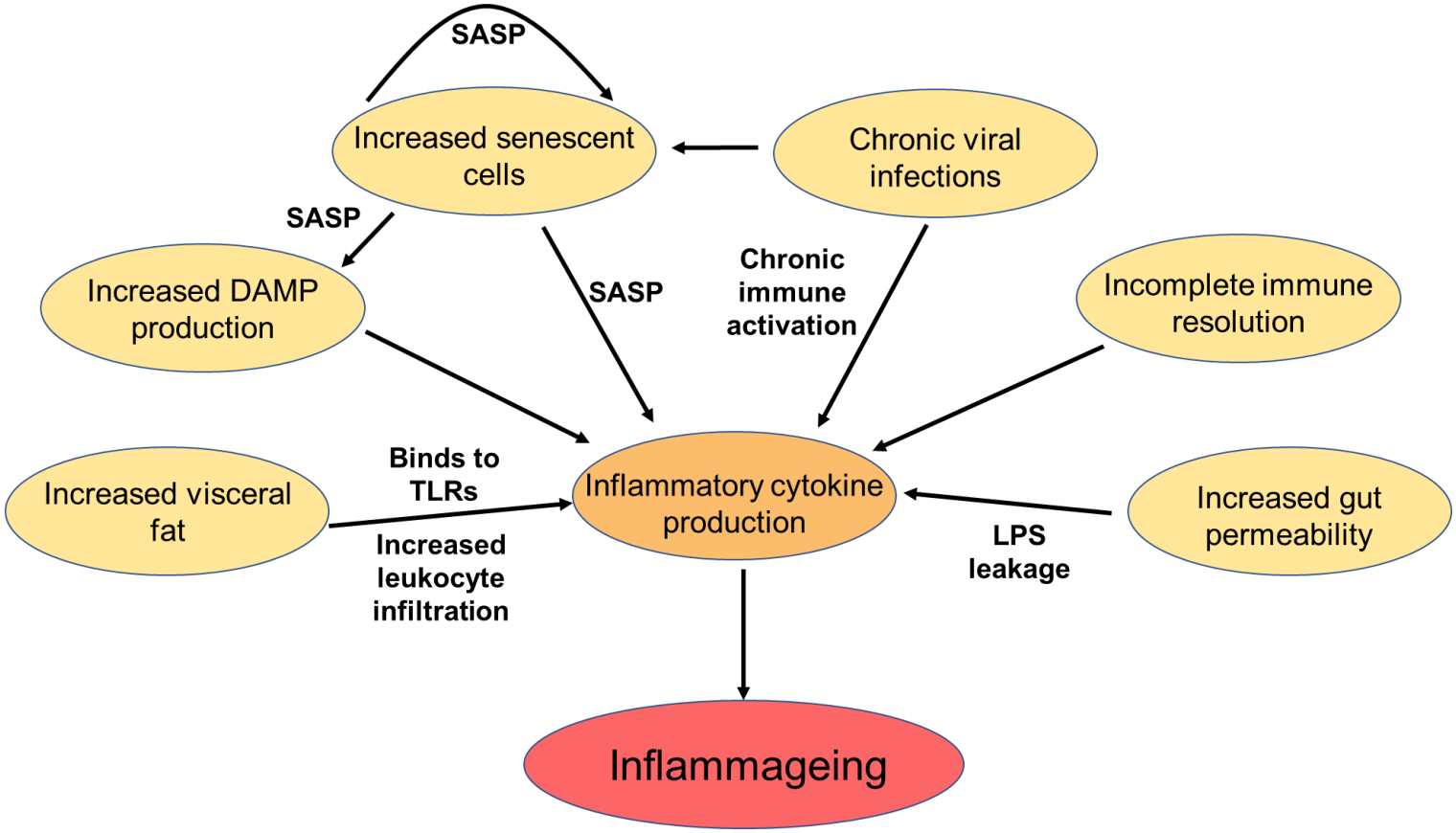

Figure 1: Potential mechanisms of inflammageing

Schematic representation of the proposed causes of inflammageing observed in the old. Increased visceral fat with associated increased leukocyte infiltration; increased DAMP production which bind to TLRs; increased senescent cell production with production of SASP which also includes DAMPs; increased gut permeability and LPS leakage and subsequent TLR activation; and finally chronic viral infection which lead to chronic immune activation. The SASP secreted from the senescent cells also has the capability to drive senescence in nearby cells, increasing the number of cells overall. All of these outcomes result in the production of inflammatory cytokines and the subsequent on set of inflammageing.

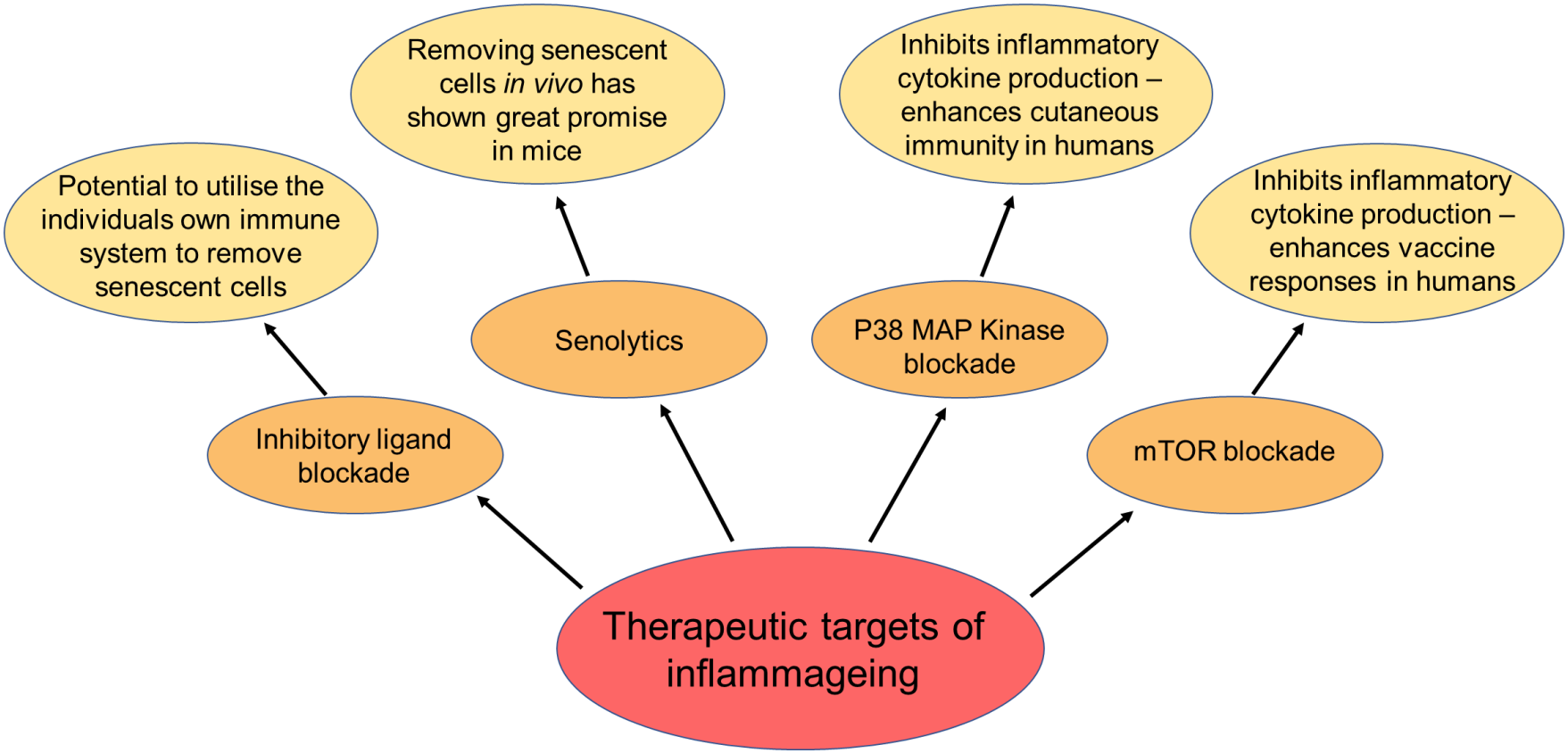

Figure 2: Schematic of the therapeutic targets of inflammageing

Schematic representation of the current therapeutic targets of inflammageing. In humans the two main targets to date have been the use of mTOR blockade to enhance vaccine responses and p38 MAP Kinase blockade to enhance antigen-specific cutaneous immunity. In mouse models senolytics have been shown to be a very promising target and are as yet untried in humans. The most untested approach is the use of inhibitory ligand blockade to enhance senescent cell clearance via the hosts own immune system.