Cellular and molecular immune markers of aging and frailty

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Chapter 8

General discussion

8.1 Summary of main findings

The immune system plays a crucial role in reaching old age in good health due to its vital importance in protection against pathogens, clearance of cellular and molecular debris, and adequate repair of cellular damage. This thesis is focused on identifying immunological markers of frailty. We provide new insights into how levels of immunological markers change over time in an aging population, how these changes possibly contribute to the chance of becoming frail, and how the immune system of frail elderly might be impaired due to dysfunctional cellular signaling through the JAK/STAT pathway.

In **Chapter 2** of this thesis, we compiled a frailty index using data from the Doetinchem Cohort Study (DCS) and calculated a frailty index score for the participants. Analyses showed that higher scores were correlated with chronological age and with an increased mortality risk independent of age, which is consistent with results of other studies (Collerton et al., 2012; K. Rockwood et al., 2011; Schoufour et al., 2017; Searle et al., 2008). Enumeration of leukocyte subsets showed an expansion of the myeloid cell lineage in frail men and women (**Chapter 2**) and signs of a remarkably consistent chronic low-grade inflammation over a time span of 20 years, as evidenced by elevated CRP levels measured repeatedly over this time period. This highlights that inflammation could be involved in the early process of becoming frail. When studying the immune cellular profile more in-depth, we observed that it differed between men and women, with higher numbers of B cells and T cells, but lower numbers of monocytes in women (**Chapter 3**). We next identified a sex-specific immune cellular profile with frailty (**Chapter 3**). This profile indicates a possibly larger role of immune changes in the process of becoming frail in women than in men. Of note were the important role of myeloid cell numbers in this profile, in particular of neutrophils and (classical) monocytes. These myeloid cells had a stronger association with frailty than T- or B cell subsets, including those typically seen

at higher ages, such as decreased numbers of naive $CD8^+$ T cells, and increased numbers of regulatory T cells and terminally differentiated $CD8^+$ T cells.

In **Chapter 4**, we analyzed aging patterns in the general population using a so-called 'healthy aging index,' a health-related score that involved fewer variables than the frailty index, was measured over time, and was validated within the Doetinchem cohort study. As expected, we saw a decrease in this score and thus signs of decline in bodily functions relatively early in the life course. In men, we identified two different aging trajectories, 'gradual aging' and 'early aging,' while only one pattern was apparent in women, largely coinciding with the pattern of 'early' aging men. Greater body mass index and lower social economic status were related to the risk of belonging to the group of 'early aging' men.

While it is known that chronic cytomegalovirus infection affects the cellular immune cell numbers greatly, especially numbers of memory T cells, we showed that the event itself of becoming infected with the virus was not strongly related to frailty (**Chapter 5**). We did not find a clear relationship of duration of CMV infection with frailty, or with the number of terminally differentiated T cells. These numbers of terminally differentiated T cells were indeed higher in CMV-infected individuals, as expected (Wertheimer et al., 2014), but did not differ clearly between short-term and long-term CMV-infected individuals. This implies that our results do not support the theory that cumulative exposure to CMV infection affects the immune system and general health in a negative manner.

Several papers have described the occurrence of chronic low-grade inflammation in older men and women, which is believed to be related to dysfunctional immune responses (Daniel Baylis et al., 2013; Claudio Franceschi et al., 2006). When studying in-depth indicators of chronic inflammation over time, we observed that IFNγ-related markers and platelet activation markers were most informative and a strong increase in those markers was strongly related to an increase in most of the other

markers (**Chapter 6**). We also observed multiple differences in inflammatory marker levels between men and women, with the differences becoming less apparent after menopause in women. The markers associated with frailty were mostly markers related to the IL-6 (signaling) pathway. In women, we also found increases in levels of sCD14, which is related to monocyte activation. Since most associations did no longer hold after adjusting for BMI, overweight could be an important factor driving the immune marker profile related to frailty.

Also signs of dysfunctional immune cellular signaling were seen with frailty, with frailer individuals showing lower cellular pSTAT responsiveness to cytokines (**Chapter 7**). These results were seen in monocytes, B cells and T cells, and with pSTAT1, pSTAT3, and pSTAT5 responses. This could be a sign that low-grade chronic inflammation exhausts the immune system, with reduced cellular JAK/STAT pathway signaling leading to inferior immune responses in frail individuals. Of interest is that dysfunctional IL-10-induced pSTAT3 signaling in men is related to both frailty and to continuously higher CRP levels over the past 15 years suggesting that regulatory signaling is impaired in frail men particularly.

8.2 Challenges in aging research

One of the main challenges in aging research is its multifactorial nature, given a vastly heterogeneous elderly population, and the lack of a uniform definition of healthy aging. Furthermore, aging research is preferably performed in longitudinal studies with individuals being followed for a prolonged period of time; one big advantage of longitudinal study designs over cross-sectional ones is that inferences about causality can be tested up to a certain extent. However, this is challenging given the longevity of humans compared to laboratory animals. One way to investigate the aging process longitudinally is by using long-running cohort studies such as the Doetinchem Cohort Study (DCS) (this thesis), in which individuals in the

general population have been followed for decades and blood samples have been collected repeatedly over a long period of time. Inherent disadvantage of retrospective longitudinal cohort studies is their observational nature and that the amount of collected data is limited. Due to this, with most associations we describe in this thesis we can speculate about causation and mechanisms but cannot prove them. Moreover, due to loss-to-follow-up and non-random drop out from the study, observational cohort studies may easily give rise to selection bias, with frail participants possibly more prone to drop out early. That said, drop out within the DCS is relatively low; more than 70% of participants continued to participate in each round (Picavet et al., 2017; Verschuren et al., 2008). Because of the unique longitudinal data in the DCS we can actually take into account within-individual variation and age-related changes in the comparisons, giving us more insight into increases in biomarker levels over time and their relationships with frailty and other health-related outcomes (**Chapter 6**). In addition, we made sure to use robust methods of statistical analysis, in particular nonparametric analysis techniques, and to control the so-called false discovery rate. The latter is especially important when investigating associations of the many immunological biomarkers with parameters of healthy aging in this thesis. In this way, we tightly controlled the risk of finding 'false' associations by maintaining a pre-defined acceptable maximum of 15% potentially false discoveries.

8.3 Immunosenescence: where does it stand in the aging process?

Chronic low-grade inflammation: harmful or protective?

The chronic low-grade inflammation that can occur in older people is generally thought to be harmful (Ferrucci & Fabbri,

2018). We indeed found associations of elevated levels of inflammatory markers in frail older people, and possibly also in people who became frail over time. It should be noted however, that the associations were weak and most of them did not hold after adjusting for BMI. Another indication that low-grade inflammation is harmful is our finding in men that continuously elevated CRP levels were associated with impaired IL10-mediated JAK/STAT signaling, a mechanism involved in anti-inflammatory regulation, and that this impaired IL10-mediated JAK/STAT signaling was also associated with frailty.

Noteworthy is that, while we indeed observed elevated levels of inflammatory markers, these higher levels were seen in only a limited set of markers. This increase in level was much less than during an acute infection and thus reflects a different kind of immunological response than the clearly pro-inflammatory response during an acute infection. It is thus important to know what the consequences are of these elevated levels of inflammatory markers: are they causing damage to the body, or are they merely a consequence of damage to the body? This is not straightforward due to the pleiotropic function of these molecules. Molecules such as CRP and IL-6 are usually described as pro-inflammatory and a strong pro-inflammatory signal can indeed inflict self-damage. However, since the levels of the inflammatory markers are all lower than during acute infection, their elevation might be necessary to induce repair mechanisms to cope with external factors that induce cellular damage such as disturbed metabolism due to an unfavorable diet, or lung damage due to air pollution or smoking. IL-6 can signal through the classical signaling pathway but also through trans-signaling after binding to sIL-6R. Most signaling of IL-6 when it is available in lower concentrations is thought to go through the classical pathway, even when sIL-6R is abundantly available (Baran et al., 2018), which is known to possess immune regulatory properties and to activate cellular repair mechanisms. Also CRP in its pentameric form, in which it is usually measured, is involved in resolution of inflammation

in low concentrations, recruiting an anti-inflammatory M2-like macrophage phenotype, and subsequently secreting factors that inhibit the complement cascade.

Thus, while markers such as IL-6 and CRP are involved in inflammation and are upregulated in acute infections, chronic elevation of these molecules is not necessarily harmful. They might fulfill a protective role in the body, being involved in damage repair mechanisms. Most problems arise when the delicate balance is lost between adequate immune reactions to evading pathogens, damage repair, and resolution of inflammation. To elucidate the role of these inflammatory markers, it is of vital importance to know how and under which conditions they were produced.

Phagocytes and their importance in the aging process

One of the most notable signs in frail elderly was the higher numbers of innate immune cells (**Chapter 2, 3**). Activated innate phagocytic cells such as macrophages can produce multiple inflammatory cytokines and thus can be a potential source of chronic low-grade inflammation (Claudio Franceschi et al., 2017). Innate phagocytic cells can also be functionally impaired in the elderly (Duong et al., 2021). Neutrophils were previously shown to have lower chemotactic migratory capacity (Wilson et al., 2020, p. 20) and decreased phagocytic functionality. Also other phagocytic cells such as monocytes and macrophages demonstrated reduced function with age. Both the phagocytic function and chemotactic migratory capacity is reduced in aged macrophages (Lloberas et al., 2019). In addition, more M2-like macrophages are seen with age, which are known to have immune regulatory and reparative functions (Duong et al., 2021; Lloberas et al., 2019). The reduced function of phagocytic cells could be a reason why more myeloid lineage derived cells are seen in frail older people (**Chapter 2, 3**), as a consequence of a 'compensatory mechanism' to maintain an adequate immune

reaction against pathogens. Impaired functioning phagocytic cells may underlie the inadequate clearance of the damage associated molecular patterns (DAMPs) that accumulate in the body with age. This can cause accumulation of these DAMPS in the body, triggering not only the production of new phagocytic cells but also the production of multiple inflammatory proteins, leading to chronic low-grade inflammation (Ferrucci & Fabbri, 2018; Claudio Franceschi et al., 2017).

Changes in numbers of myeloid cells can also be due to hematopoietic stem cells being more inclined to produce myeloid progenitors, which may occur in older people with low-grade inflammation (Kovtonyuk et al., 2016). This shift of hematopoietic stem cells towards the myeloid lineage is possibly driven by plasma cells in the bone marrow, since plasma cells in bone marrow can produce pro-inflammatory cytokines which also influence myelopoiesis (Pioli et al., 2019). Thus, innate phagocytic cells clearly play an important role in the aging process, being pivotal for early defense against pathogens but also for damage repair mechanisms, and being probably involved in chronic low-grade inflammation seen in older people.

Sex differences in immunosenescence

Our finding that several immune cell subpopulations differ in abundancy between the sexes is in line with previous studies (van der Heiden et al., 2016). Our results also suggest that the skewing of hematopoietic stem cells towards production of myeloid cells with frailty, is more pronounced in women than in men. An explanatory factor in these sex differences could be sex hormone levels. Immunological homeostasis in women may involve a different balance between $CD4^+$ and $CD8^+$ T cells than in men, with estrogen levels known to be involved in lymphocyte development and in particular CD4 T cell proliferation (Bupp, 2015; Pennell et al., 2012). We suggested that the hormonal shift during menopause could explain why the immune marker profile becomes more similar between men and

women after the fifth or sixth decade of life (**Chapter 6**). This would be in agreement with other studies suggesting that sex hormone levels play a major role in this process (Bupp, 2015; D. Furman et al., 2014). In addition, immune function differs between the sexes, with women showing stronger responses to antigens and a stronger tendency to develop autoimmune diseases (Bupp, 2015; Gordon & Hubbard, 2019). Testosterone is also thought to impact this process since it is believed to have anti-inflammatory properties (D. Furman et al., 2014). Our results might also give more clues as to why there is a so-called sex-frailty paradox, namely that women generally tend to be frailer, yet live longer than men (Gordon & Hubbard, 2019).

Cytomegalovirus: a less important factor in healthy aging than previously thought

CMV, being a chronic infection, is known to continuously influence cellular immunity, with CMV-infected individuals showing especially more terminally differentiated T cells in peripheral blood (van de Berg et al., 2008; Wertheimer et al., 2014). Therefore, CMV infection was long thought to play a key role in immunosenescence due to its role in constantly engaging the immune system. This constant engagement is thought to exhaust the T cell pool, negatively affecting general immune responses, and to lead to a poorer health status gradually over time. We did confirm previously found associations of CMV infection with the occurrence of cardiovascular diseases (**Chapter 5**), which might be due to increased plaque formation around CMV-infected endothelial cells. Furthermore, we observed that levels of CMV-specific antibodies increased with age, and that people who became infected at older age, had antibody levels similar to their age-matched peers. However, we did not find strong evidence that longer duration of CMV infection is related to altered functionality of CMV-specific T cells, to higher levels of CMV-specific antibodies nor to differences in general health or (an increase in) frailty. These results

are in agreement with the latent nature of the CMV virus infection, with more CMV reactivation and severe CMV disease seen in people with impaired immune reactions. They suggest that the continuous reactivation of CMV and the accumulation of terminally differentiated T cells in CMV infected humans are not exhausting the immune system in a detrimental way. The results are also in agreement with the fact that there are multiple countries where almost everyone is chronically infected with CMV while the life expectancy is still high. This indicates that if there is a detrimental effect of long-term CMV infection on the immune system, the effect on general health or frailty is probably only minor. In summary, long duration of CMV infection does not necessarily impair immune functions, and the 'burden' of a long exposure to CMV infection appears to be limited, although reactivation of CMV may occur more often in people that are frail and have an impaired immune system due to the opportunistic nature of the virus, possibly leading to more CMV disease.

Is there an immune biomarker profile related to frailty?

We were able to identify multiple immune markers that were related to frailty in this thesis. It became clear that the most profound changes with frailty were seen in the myeloid cell lineage, and that IL-6 pathway related markers were consistently elevated in the frail population, with stronger associations in women. While these are still only associations with frailty and therefore do not prove a causative relationship, the fact that multiple immune markers were elevated for a prolonged period of time in frail individuals makes it plausible that these markers' elevations preceded frailty or at least are involved at an early stage in the process of becoming frail. In line with this, we observed that levels of, again, the IL-6 pathway related marker C-reactive protein were elevated during a prolonged period of time in women that were healthy at baseline and became frailer

over time. Thus, we did indeed identify an immune profile related to frailty, with more innate phagocytic cells, and higher levels of IL-6-pathway related markers. Our results are compatible with the theory that chronic low-grade inflammation precedes frailty and leads to a higher risk of becoming frail (Figure 8.1). It is to be noted, however, that the few other longitudinal studies investigating this subject did not find such a relation, as was described in a recent meta-analysis (Soysal et al., 2016). This might be due to either a shorter follow-up time in previous studies, or less frequent measurements being available. However, we found the predictive power for frailty, using a combination of immune parameters, to be weak, which thus limits the use of the inflammatory markers as biomarkers to predict frailty in individuals. This weak predictive power shows the challenge of identifying strong predictors of frailty given the multifactorial nature of aging and the many ways that lead to frailty besides an altered immunological state.

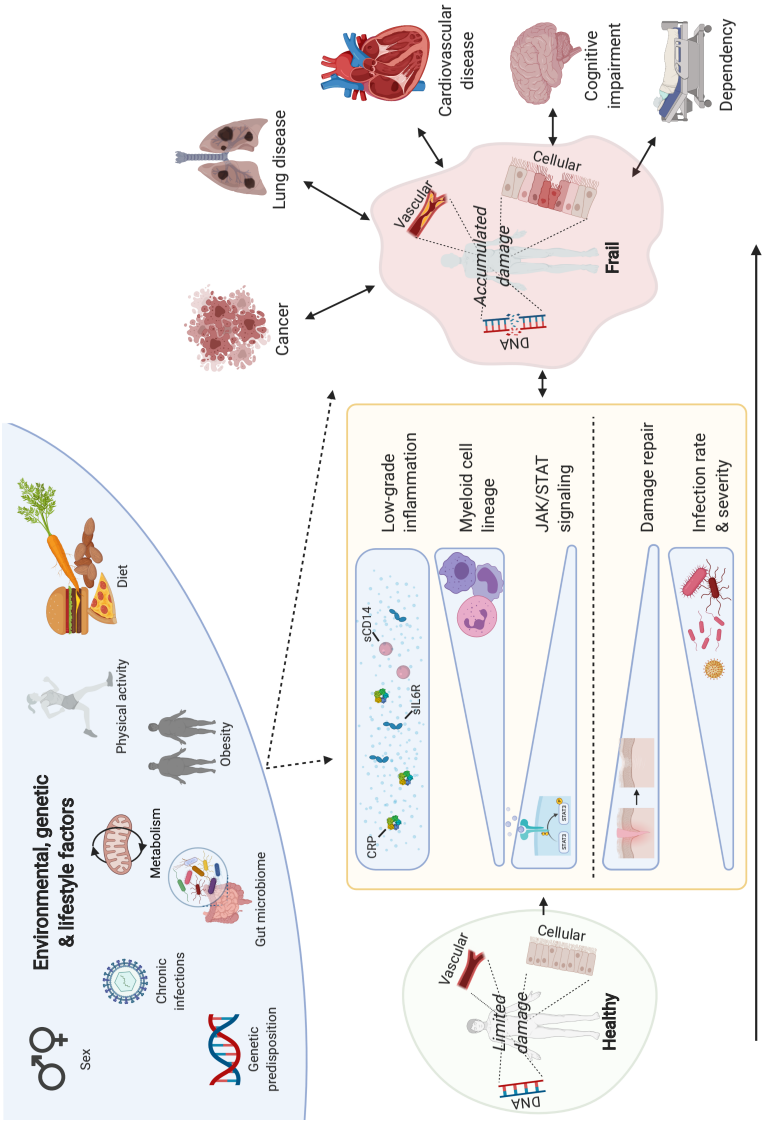

Figure 8.1: *(see next page)*

Figure 8.1 *(previous page)*: Immunological aspects that might precede frailty, including environmental, genetic & lifestyle factors that are of influence in this process. Important immunological factors are a chronic low-grade inflammation (**Chapter 2, 6**), an increase in myeloid cell numbers (**Chapter 2, 3**), and reduced cellular cytokine responsiveness due to impaired JAK/STAT signaling (**Chapter 7**). Other immune system-related factors are reduced cellular damage repair and increased infection rate and severity. Several environmental, genetic & lifestyle factors are of influence of this process of becoming frail. The process of becoming frail is complex and probably not unidirectional, with most factors involved also influencing other ones. Frail people are more prone to develop major diseases and symptoms such as cancer, lung diseases, cardiovascular diseases, or cognitive impairment all adding to their dependency on health care.

In which way are immunosenescence and frailty connected?

We aimed to clarify which aspects of an aging immune system are most relevant in remaining healthy into old age. We think that this thesis shows that, indeed, a 'disbalanced' immune system plays a role in the process of becoming frail. We found that people who were frail had high levels of IL-6 related inflammatory markers, high numbers of phagocytic innate immune cells, and defective cellular signaling through the JAK-STAT pathway (as evidenced by inadequate responses to cytokines). These individuals may have 'underperforming' immune responses with inadequate cellular- and DNA damage repair, more frequent and severe infections, and inability to resolve inflammatory reactions (Figure 8.1), leading to an accumulation of damage in the body and a higher chance of becoming frail.

Important factors in this process could be related to lifestyle, such as being overweight or obese. Adipocytes, increased in number in obesity, are known to secrete IL-6 related pro-inflammatory cytokines (David Furman et al., 2019) and to recruit innate phagocytic cells such as macrophages which contribute to the production of pro-inflammatory cytokines (Ghigliotti et al., 2014). An immunological phenotype of elevated IL-6 related markers and elevated numbers of phagocytes is consistent with what we found in frail older individuals. Also

consistent with this phenotype is that adiposity is related to dysfunctional pSTAT signaling (Dodington et al., 2018). Thus, adiposity could be one factor explaining the development of a frailty-related immune profile. Also diet might play a role in this process, which, amongst others, is believed to influence the inflammatory status by altering the intestinal flora, with an unfavorable diet causing an 'intestinal dysbiosis' (David Furman et al., 2019). A proper, varied diet is also important to ingest enough micronutrients important for immune function, such as vitamin C, vitamin D and zinc (Gombart et al., 2020). Another important lifestyle-related factor that could influence the relationship between immunosenescence and frailty is physical activity. While exercise can cause muscle damage which induces an inflammatory response shortly after the injury, probably due to cell damage repair mechanisms (Metsios et al., 2020), it is commonly believed that exercise has a long-term anti-inflammatory effect (Simpson et al., 2015). In line with this, physically active people were shown to have consistently lower levels of inflammatory markers over longer periods of time (Hamer Mark et al., 2012). Lastly, genetic factors may also play a role in developing low-grade inflammation or in the process of becoming frail, with some people being more prone to develop chronic low-grade inflammation than others. Several genetic polymorphisms are known to be related to levels of pro-inflammatory markers such as CRP (Ferrucci & Fabbri, 2018). The relationship of immunosenescence with frailty and with the process of becoming frail is complex and probably not one directional (Figure 8.1). Someone may become frail after a specific event, such as a bone fracture and become dependent on care without the immune system being directly involved. Environmental and lifestyle factors might both induce and catalyze a decline in the immune system's functioning. Also, cellular damage itself could lead to accumulation of phagocytic cells such as neutrophils, something seen often in the frail population. Thus, higher numbers of specific immune cells could be a physiological sign of the body coping with cellular damage,

and is not necessarily a sign of a dysfunctional immune system. Whether higher numbers of phagocytic cells in elderly directly affect the immune system's ability to adequately deal with infections remains to be investigated.

8.4 Implications and future perspectives

Towards predicting frailty in older adults

We identified immunological markers characteristic of frailty (this thesis). The markers that we found give important insight in the aging process and in immunosenescence. Despite the fact that we used long follow-up of participants, the evidence for inflammatory markers to actually predict which people will become frail is sparse. However, our set of inflammatory markers was certainly not exhaustive and can be extended to include other potentially useful markers to predict frailty, for instance those related to monocyte and neutrophil function and activation, or those related to the IL-6 pathway. Furthermore, since we did find several associations, they could probably help to identify people at risk of becoming frail when combined with other meaningful clinical data, such as body mass index/waist circumference, and physical activity. When in future studies other biomarkers of frailty are found, they could be combined with inflammatory markers to create a better prediction of people being at risk of becoming frail.

Next steps to consider in investigating immunological aspects of healthy aging

Steps that could be taken in further investigation to find out which mechanisms are involved are the following. One could investigate more specific health deficits, to unravel which of these are most strongly associated with inflammatory markers. Another important aspect to unravel is the exact phenotype of the innate immune cells that are elevated in numbers in the frail

older individuals. A more in-depth analysis of phenotypic and functional characteristics of the frailty associated neutrophils and monocytes , preferably sampled at multiple time points during the day due to the short lifespan of these cells, could help to unravel the reason of their increase in numbers: do expanded numbers of these cells merely reflect the greater abundance of damaged cells that need to be cleared in frail individuals? Or are they there because their function is impaired, and more of them are needed to maintain adequate protection against pathogens? These questions remain to be answered and could be addressed in follow-up studies.

Focusing on changes of hematopoietic stem cells in future studies could also help to unravel which immunological changes are relevant for healthy aging, since these are thought to play an important role in this process (Kovtonyuk et al., 2016). With bone marrow biopsies, studies can help to differentiate whether changes in hematopoietic stem cells are important drivers of the observed immunological changes with aging and frailty that we observed. In future studies it can also be helpful to investigate biopsies of (visceral) adipose tissue. This way, the amount of macrophages infiltrating adipose tissue can be linked to low-grade inflammation. In combination with bone marrow biopsies, this will help to identify the most important causes of low-grade inflammation.

A technique that could be particularly useful for more in-depth analysis of peripheral blood samples and tissue biopsies (e.g. bone marrow, visceral fat) is single-cell RNA sequencing. This technique and its use are still advancing rapidly due to novel computational strategies (Wen & Tang, 2018) and decreasing costs, respectively. If used properly, it can reveal complex differences in immunological functioning and signaling in single cells that would otherwise be impossible to detect (X. Tang et al., 2019). Due to the techniques' complexity, sequencing should in the first place be done on cross-sectional samples. When it has been identified which pathways are affected in frail older people, researchers can ultimately focus on

these pathways and study how they become altered over time in longitudinal cohort studies.

Another important question still outstanding is how changes of immune markers relate to sensitivity to and severity of infections. An important theory discussed in this thesis is that people with impaired JAK/STAT signaling will demonstrate hyporesponsiveness to cytokines and therefore will be more prone to develop severe infections, leading to a higher chance of becoming frail. It is known that the JAK-STAT pathway is important for cytokine signaling and that complete loss-of-function mutations can lead to serious life-threatening diseases such as severe combined immunodeficiency (O'Shea et al., 2015). Less severe loss-of-function mutations have also been described in many STAT signaling pathways and are generally related to increased susceptibility to infections. In the case of STAT1-related mutations, these are mainly mycobacterial but also viral infections (Dupuis et al., 2003; Sancho-Shimizu et al., 2011), due to lower responses to IFNγ and IFNα (Chapgier et al., 2006; Vairo et al., 2011). Loss-of-function mutations in STAT3 signaling cause the hyper IgE syndrome, which is characterized by candidiasis, bacterial infections, and reactivation of chronic herpes virus infections (O'Shea et al., 2015). Finally, loss-of-function mutations in STAT5 signaling can also lead to severe bacterial or viral infections and to defective growth hormone signaling (Herrington et al., 2000). These studies show the importance of STAT signaling for an adequate immune reaction to infection. Future studies should investigate whether the lower pSTAT responses in frail individuals (this thesis) indeed are clinically meaningful and are related to a higher risk of infection. This could be done, for instance, by investigating whether pSTAT responsiveness is related to vaccine effectiveness, or to severity of disease. Furthermore, due to the clinical relevance of pSTAT responsiveness, it would be valuable to develop tests that are easier to implement in a broad clinical setting so that research in this field can advance faster.

Targeting immunosenescence with lifestyle

The first interventions that should be considered to target immunosenescence should be related to improving lifestyle, such as increasing the amount of regular physical activity, improving diet, and reducing overweight. Interventions based on prevention should always be considered first since their beneficial effects have been described extensively, and they are well-known to positively impact healthy aging. In this thesis we have shown that overweight should be taken into account when studying immunosenescence since BMI can influence multiple immunological parameters, and possibly also influence function of immune cells. As discussed in recent review articles, several studies have already been performed that show positive effects of exercise intervention (Metsios et al., 2020) and weight loss (Bianchi, 2018) on chronic low-grade inflammation.

Targeting immunosenescence with drugs: where do we stand?

Since the function of innate phagocytic cells might be reduced in frail elderly, improving the function of these cells could be beneficial. A possibly important aspect of impaired neutrophil function in older people could be a higher expression of phosphoinositide 3-kinase (PI3K) (Naccache & Lefebvre, 2014). Early studies have shown improvement in neutrophil function and chemotactic capacity in vitro by using PI3K inhibitors (Naccache & Lefebvre, 2014; Wilson et al., 2020, p. 202). Phagocytic cells such as neutrophils are of vital importance for early defense against pathogens and improvement in their function and in their efficiency of chemotactic migration could potentially have a large impact on general health in vulnerable older people.

Specific techniques targeting inflammation could also be used to improve vaccine effectiveness in the elderly. In one study, administering anti-inflammatory drugs that block p38 signaling reduced the amount of low-grade inflammation and

improved the immune response to varicella zoster virus antigen in the skin (Vukmanovic-Stejic et al., 2018). The result of this preclinical study is promising since it might be an option to boost vaccine effectiveness in elderly, and because the drugs can be given for a short period of time to be effective.

Another possible way to combat immunological decline in the elderly could be to target senescent cells, which are more abundant in the elderly and might be partially responsible for low-grade inflammation due to their production of inflammatory cytokines and chemokines. These cells could be targeted directly by using so-called 'senolytic' drugs (Childs et al., 2017), or indirectly by suppressing the senescent associated secretory phenotype with, for example, NF-kB inhibition, or inhibition of target of rapamycin complex 1 (mTORC1), which showed promising results in preclinical studies (Childs et al., 2017; Song et al., 2020).

Also targeting cellular signaling through the JAK-STAT pathway could be an option for early intervention, since this signaling pathway might be impaired in elderly (Shen-Orr et al., 2016) and in frailty (**Chapter 7**). JAK inhibitors are already being used successfully for treatment of auto-immune diseases such as ulcerative colitis (Sandborn et al., 2017) and rheumatoid arthritis (Heijde et al., 2019), and it could be reasoned that they are helpful to combat low-grade inflammation in elderly. Since a lower IL-10 induced STAT responsiveness was seen in several leukocyte lineages of frail men (**Chapter 7**), and also was related to chronic low-grade inflammation in men, it could be that these individuals are less capable of resolving inflammation by means of the immune regulatory signaling of IL-10. Some preclinical studies suggest that low-grade inflammation in older people can be reduced with JAK inhibitors. (Febvre-James et al., 2020; Xu et al., 2015). However, JAK inhibitors have an immune suppressive effect, posing restrictions for its use in certain patients such as the ones that are immune compromised or have cancer. It should also be investigated whether these drugs can boost vaccine effectiveness by

reducing low-grade inflammation, just before vaccination in a similar manner as with p38 inhibitors (Vukmanovic-Stejic et al., 2018) The JAK inhibitors are also currently being investigated for their use of preventing a "cytokine storm" in severe COVID-19 disease, although with mixed results. Future studies could help to elucidate whether testing cellular pSTAT responsiveness could help to identify patients that benefit most from JAK inhibitor therapy.

8.5 Concluding remarks

Clarifying the role of the immune system in aging and in frailty is of vital importance to elucidate why some people reach old age in good health and others do not. We aimed to identify immunological biomarkers of aging and frailty, and to clarify which changes in inflammatory markers over time were clinically important signs of immunosenescence in elderly. Furthermore, we speculated which immunological mechanisms are important in low-grade inflammation, with cellular cytokine responsiveness possibly being important in this process, also as a potential target for treatment of immunosenescence in the future.

We indeed found an immunological profile related to frailty, showing dysregulation of the innate immune system and impaired cellular cytokine responsiveness in frail individuals. We also provide evidence for sex-specific differences, with women showing more and stronger associations of cellular and molecular immune markers with frailty. This highlights the importance of investigating men and women separately in aging research. The contribution of CMV serostatus and duration of CMV infection to the frailty-related immune profile appears to be limited. One of our main hypotheses was that low-grade inflammation precedes the onset of frailty. Although no definite conclusions can be drawn based on our data, we indeed found evidence for this hypothesis.

In the complex and multifactorial aging process, results in this thesis inform and show how the immune system is involved in the aging process, with novel (sex-specific) immune markers being identified that can complement already available clinical markers and signs related to healthy aging . We hope that our study will prompt future studies to employ novel technologies in well-described longitudinal population cohorts of aging to reveal predictors of frailty in men and women.