

Impaired JAK-STAT Pathway Signaling in Leukocytes of Frail Elderly

Leonard Daniël Samson (✉ leon.samson@rivm.nl)

National Institute for Public Health and the Environment <https://orcid.org/0000-0002-6252-7639>

Peter Engelfriet

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

W. M. Monique Verschuren

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

H. Susan J. Picavet

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

José A. Ferreira

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

Mary-lène de Zeeuw-Brouwer

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

Anne-Marie Buisman

National Institute for Public Health and the Environment: Rijksinstituut voor Volksgezondheid en Milieu

A. Mieke H. Boots

University Medical Centre Groningen: Universitair Medisch Centrum Groningen

Research

Keywords: Immunosenescence, frailty, JAK-STAT pathway, immune function, phospho-flow cytometry, chronic low-grade inflammation

Posted Date: October 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-970801/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Immunity & Ageing on January 17th, 2022. See the published version at <https://doi.org/10.1186/s12979-021-00261-w>.

Abstract

Background: Elderly often show reduced immune functioning and develop chronic low-grade inflammation. Why some elderly are more prone to become frail is unknown. We investigated whether frailty is associated with differences in cytokine signaling through the JAK-STAT pathway in leukocytes of 34 individuals aged 65-77 years. In addition, we investigated how this relation is affected by chronic low-grade inflammation during the previous 20 years. Cytokine signaling was quantified by measuring intracellular STAT1, STAT3, and STAT5 phosphorylation in monocytes, B cells, CD4⁺ T cells and CD8⁺ T cells upon stimulation with IL-2, IL-6, IL-10, IFN α and IFN γ , using phospho-flow cytometry. Plasma inflammatory markers had been measured repeatedly in the same individuals over the previous 20 years. Frailty was assessed as a score on a frailty index.

Results: We found that lower cytokine-induced pSTAT responsiveness in the various cell subsets was seen with higher frailty scores in both men and women, indicative of dysfunctional pSTAT responses in frailer individuals. Associations differed between men and women, with frailer women showing lower pSTAT1 responses in monocytes and frailer men showing lower pSTAT5 responses in CD4⁺ and CD8⁺ T cells. Notably, lower IL-10-induced pSTAT3 responses in men were related to both higher frailty scores and higher CRP levels over the past 20 years. This might indicate poor resolution of low-grade inflammation due to defective regulatory pSTAT signaling in older men.

Conclusions: Our results emphasize the importance of preserved JAK-STAT pathway signaling in healthy aging and reveal cellular pSTAT levels as a candidate biomarker of frailty.

Introduction

Adequate functioning of the immune system is thought to be a pivotal factor in the healthy aging process (1). Older people are known to have diminished vaccine responses (2, 3), inadequate reactions to combat new infections such as COVID-19 (Glynn & Moss, 2020), and weaker immune mechanisms for clearing (pre)malignant cells (4). An important sign of immune dysregulation is the presence of a 'sterile' low-grade chronic inflammation, more often seen in older individuals (5–7). It is thought that this low-grade inflammation coincides with a reduced functioning of the cellular immune system, especially that of macrophages, leading to a poor clearance of accumulating tissue debris and senescent cells from the body (8). In addition, senescent cells could directly contribute to chronic low-grade inflammation since they produce multiple inflammatory cytokines (known as the Senescence Associated Secretory Profile, SASP), and these cells are more abundant in older people (9).

An important factor relevant to reduced immune responses in older people could be impaired cellular cytokine signaling. Many cytokines are known to signal through the Janus kinase-Signal Transducer and Activator of Transcription proteins (JAK-STAT) pathway. In this pathway, cytokines (mainly interleukins and interferons) bind to surface receptors causing a chain reaction which ultimately leads to the intracellular phosphorylation of STATs. This, in turn, can induce transcription of genes leading to

adequate immune responses (10). Loss-of-function mutations within the JAK-STAT pathway are related to life-threatening diseases such as severe combined immunodeficiency (11), and to increased susceptibility to and severity of infections (12, 13). Conversely, overactivation of the JAK-STAT pathway is also linked to dysfunctional immune responses. This is currently of particular interest since JAK inhibitors have recently been developed that were shown to be effective in the treatment of auto-immune diseases such as ulcerative colitis (14) and rheumatoid arthritis (15).

JAK-STAT signaling was found to be impaired in older people, with reduced signaling (16), and higher baseline cellular STAT activation (16, 17). Also, results of preclinical studies suggest that low-grade inflammation in older people can be reduced with JAK inhibitors. (18, 19). The immune system in elderly may also be dysregulated due to inadequate responses to triggering of pattern-recognition receptors, such as toll-like receptors (TLR). These receptors can recognize pathogens or parts of pathogens and activate immune cells, which can in turn produce pro-inflammatory cytokines. Diminished TLR responses have been reported with higher age in most studies (20, 21) although not in a study that investigated strictly healthy older participants (22).

To better understand the role of cellular signaling in the aging process, it is important to know if reduced cellular immune responses are seen in all elderly or only in those that are frail. Frailty can be assessed by means of a frailty index score; people with a high score have been shown to have an increased risk of 'adverse' life events (e.g. injury) and a reduced capacity to recover from these events (23, 24).

In this study we investigated whether diminished immune cellular responses in older men and women are related to frailty and if impaired cytokine signaling is related to more pronounced chronic low-grade inflammation. We analyzed cytokine-induced cellular signaling through the JAK-STAT pathway and studied cellular activation through the TLR pathway in 34 individuals selected from a Dutch longitudinal population-based cohort study. The presence of chronic low-grade inflammation was evaluated by analysis of 18 different inflammatory markers in the same individuals (at 5 time points) longitudinally over the past 20 years.

Results

Study population characteristics

The 34 participants were 65-75 years old (Figure 1), selected from the Doetinchem cohort study (DCS), a longitudinal study that started in 1987 with the participants being followed up ever since (25, 26). CMV seropositive individuals were excluded in order to avoid CMV infection being a confounder in the study. The median frailty index score of the participants was 0.09, ranging from 0 (0/36 deficits present) to 0.4 (14/36 deficits) (Table 1). The mean BMI level was 26.9 kg m⁻² and ranged from 20.8-35 kg m⁻². The frailty index score (see **Table S1** for frailty index components) and BMI levels did not differ significantly between men and women (**Figure S1**).

Table 1
Baseline characteristics of the study population (n=34)

Women, %(n)	52.9 (18)
Age, yrs.	69.4 (SD: 2.6, range: 65.4 - 74.1)
BMI, kg m ⁻²	26.9 (SD: 3.4, range: 20.8 - 35)
Frailty index score	0.09 (0.18, range: 0-0.4)
CMV-, %	100
Frailty index score: health score (median + interquartile range) enumerating 36 health deficits, theoretically ranging from 0 (no deficits) to 1 (all deficits). BMI and age are in mean + standard deviation. CMV-seronegative study participants were selected for this study.	

Cytokine-induced pSTAT responsiveness within different immune cell lineages

We used a broad panel of experimental conditions to quantify immune cell cytokine responses by separately stimulating PBMCs with IL-10, IFN γ , IL-6, IL-2, or IFN α , and subsequently measuring phosphorylated STAT1, STAT3, and STAT5 (pSTATs) in monocytes, B cells, and CD4⁺ and CD8⁺ T cells (Figure 1), using phosho-flow cytometry (Figure S2). Baseline (unstimulated) levels of pSTAT1, pSTAT3, and pSTAT5 were higher in monocytes than in the other cell subsets, and those of pSTAT5 were higher than those of pSTAT1 and pSTAT3 in both CD4⁺ and CD8⁺ T cells. (Figure S3, light grey density plots). The baseline pSTAT levels did not differ significantly between men and women (Table S2). Monocytes showed relatively high induced pSTAT levels (after stimulation), besides high baseline pSTAT levels. As expected, not all experimental conditions induced responses of pSTAT1, pSTAT3 and pSTAT5; therefore we selected the conditions showing a 'clear' response upon stimulation (defined as a median fold change ≥ 2) for further analysis (Figure 2, Figure S4, 20 out of 60 stimulation conditions). Most of such responses were induced by IFN α . Other responses described in literature that we observed were IL2-STAT5 responses in T cells (11), IFN γ -STAT1 responses in monocytes and B cells (11), and IL6- and IL10-induced STAT3 responses in monocytes and CD4⁺ T cells. IL6 also induced STAT1 in CD4⁺ T cells. The highest responses were seen in CD4⁺ and CD8⁺ T cells after stimulation with IL-10, with a fold change in pSTAT3 levels of 11.9 and 15.2, respectively (Table S3). When testing for sex-specific differences, we did not observe differences in baseline pSTAT levels between men and women, but we did find that women had higher pSTAT3 responses to IL-10 stimulation in CD4⁺ T cells and to some extent also in CD8⁺ T cells, although the differences were small (Table S4, Figure 2). Next, we examined how cell subset numbers differed between the sexes and with frailty in this population, since a difference in cell numbers might be a confounder in our analyses. We observed that women on average had higher numbers of B cells but lower numbers of monocytes than men (Figure S5A). In line with our previous data (27), monocyte numbers in women, but not men, were higher with higher frailty index scores (Figure S5B).

Frailty is associated with lower cellular pSTAT responsiveness

We related frailty score to baseline pSTAT levels and found a negative association between a higher frailty score and baseline pSTAT1 in monocytes of men but not women (Figure 3A and B). When we investigated the relationship of frailty with the cellular pSTAT cytokine responsiveness (upon cytokine stimulation), most of the observed associations were negative, i.e. negative correlations, and differed between men and women (Figure 4A).

Associations of pSTAT1 responsiveness with frailty were mainly seen in women (Figure 4A,B). pSTAT1 responses of monocytes (Figure 4B, $\rho=-0.53$ after IFN α and $\rho=-0.52$ after IFN γ stimulation) and of CD4⁺ T cells (Figure 4B, $\rho=-0.64$ after IL-6 stimulation) were found to be weaker in frailer women. In contrast, pSTAT1 responses of monocytes were found to be stronger in frailer men ($\rho = 0.60$) after IFN γ stimulation). Negative associations of pSTAT3 responsiveness with frailty were seen in B cells of men and T cells of both men and women, with most associations observed in men (Figure 4A,C). IFN α -induced pSTAT3 responses were negatively related to frailty in CD4⁺ T cells of women but not in men, and to CD8⁺ T cells of both men and women. In men, other negative relationships were found between IL-10-induced pSTAT3 responses and frailty in B cells, CD4⁺ T cells, and CD8⁺ T cells ($\rho=-0.65$, $\rho=-0.59$, $\rho=-0.66$, respectively). Lastly, associations of pSTAT5 responsiveness with frailty were only seen in T cells of men (Figure 4D). Negative associations in men were found of IFN α -induced pSTAT5 responses with frailty in CD4⁺ T cells ($\rho=-0.69$) and of IL-2 induced pSTAT5 responses with frailty in CD4⁺ and CD8⁺ T cells ($\rho=-0.71$ and -0.82 , respectively).

It is known that BMI can influence the inflammatory profile and that BMI is generally higher in frail individuals. When associations were directly tested between BMI and the cellular pSTAT responses, they had similar direction as with frailty, but none of the associations was strong enough with an acceptable false discovery rate (Figure S6).

Thus, in general multiple associations of pSTAT responsiveness with frailty were seen, with overall lower cytokine induced cellular responsivity in frailer individuals. The direction of the associations was mostly similar in men and women. However, the strength of the associations differed, with more associations found in men. Furthermore, impaired pSTAT1 responsiveness of monocytes was found in frailer women and impaired pSTAT5 responsiveness of CD4⁺ and CD8⁺ T cells in frailer men.

Defective regulatory pSTAT signaling in men with chronic low-grade inflammation

Since chronic low grade inflammation may impact cellular responsiveness, we next explored whether cellular pSTAT levels were related to any of 18 different inflammatory markers that were measured longitudinally, namely with 5-year intervals over the past 20 years in the same individuals. Cumulative exposure to low-grade inflammation over this time period was estimated, by calculating the area under

the curve of every inflammatory marker for each individual. We then related the AUC's to their cellular pSTAT responses. A clustering algorithm identified three clusters of markers based on Spearman's ρ values (Figure 5A). As the heatmap shows, positive correlations were more prominent in women, which contrasted with the more prominent negative ones in men, especially in clusters 2 and 3. Higher CRP levels in the past 20 years in men were associated with lower IL-10 induced pSTAT3 responses (Figure 5A,B), which might indicate defective regulatory IL-10 signaling in men. This association was the one with the highest ρ value ($\rho=-0.85$) and the only one below the pre-set false discovery rate threshold. Other high ρ values ($\rho > 0.7$ or $\rho < -0.7$) were also seen between CRP levels and IL-10 induced pSTAT3 responses in B cells in men ($\rho=-0.71$) (Figure 5B). In women the highest ρ value was found for the relationship between sGP130 levels and IL-6 induced pSTAT3 CD4⁺ T cell responses ($\rho = 0.72$). Of note is that, while frailty was related to several pSTAT responses induced by IL-10 and IL-6, no association was found of pSTAT responsiveness with IL-10 or IL-6 levels in the circulation. Thus, only in men associations were found between pro-inflammatory marker levels in the past 20 years and pSTAT responses, possibly showing signs of a defective IL-10 signaling.

No associations found between frailty and immune cell cytokine production after TLR stimulation

We also investigated if cytokine production is different in frail individuals after activation of specific pattern recognition receptors that are thought to be involved in inflammation. After PBMCs were stimulated for 24h with agonists of either Toll-like receptor (TLR)4 (lipopolysaccharide, LPS), TLR7/8 (Resiquimod, R848) or TLR9 (CpG ODN), we measured production of the cytokines IFN α , IFN γ , TNF α , IL-10, IL-1 β , IL-8, MCP-1, CXCL10 and of sGP130. Stimulation with LPS and R848 resulted in enhanced cytokine production for most cytokines, while CpG ODN stimulation generally resulted in no or low cytokine production. Levels of IFN α were below detection limit after 24h stimulation for the majority of the samples. A selection was made of 20 'clear' responses to TLR-ligands ('clear' defined as median >2-fold change in cytokine production compared to control sample), which were used for further analyses (Figure 6A,B). Cytokine production after TLR activation of PBMCs did not differ between men and women when testing either cytokine concentrations after stimulation (Figure 6A) or fold change in concentration (Figure 6B, **Table S9**). Regarding correlations between frailty and PBMC cytokine production conditions, more negative Spearman's ρ values were found in men than in women, but no association with frailty was found in either men or women (Figure 6C, **Table S10**).

Discussion

Our main finding is that frailty in older individuals of 65-77 years coincides with lower cellular pSTAT responsiveness. Negative associations between pSTAT responsiveness and frailty were found in both men and women. More and stronger associations were found in men, with signs of lower immune regulatory (IL-10 induced) pSTAT3 responses in frailer men. These lower responses in frailer men were related to higher levels of CRP in the past 20 years. Our results extend previous knowledge that showed

lower pSTAT responses at higher age and in cardiovascular dysfunction (16), and underline the importance of the JAK-STAT pathway in frailty and, possibly, immunosenescence.

To our knowledge, no other studies related cellular pSTAT responsiveness to frailty. Lower pSTAT responses were previously found to be related to markers of cardiovascular disease and to chronological age (16). More specifically, IFN α -induced pSTAT3 in CD8⁺ T cells and pSTAT5 in CD4⁺ T cells were negatively associated with age. Our data extend these findings by showing that IFN α -induced pSTAT3 responses of CD8⁺ T cells were also negatively associated with frailty in both men and women and that IFN α -induced pSTAT5 responses in CD4⁺ T cells were negatively associated with frailty in older men. While previous studies found higher levels of baseline pSTAT levels at higher ages (16, 17) and with higher CRP levels (16), we found an opposite relationship with frailty instead, namely lower baseline pSTAT1 levels in monocytes of frailer men. Given these previous findings, this was unexpected since chronic low-grade inflammation is seen more often in frail individuals (28–30). Thus, it is unclear how this should be interpreted with regard to the theory that immune cells of frail older people show higher baseline JAK-STAT pathway activation. We speculate that baseline STAT phosphorylation is reduced in immune cells of frail elderly due to these cells being continuously triggered by chronic low-grade inflammation, leading to cellular exhaustion; further studies are needed to confirm this hypothesis. On the other hand, impaired cytokine signaling may cause reduced immune responses, leading to accumulation of damage-associated molecular patterns and thereby induction of chronic low-grade inflammation. Thus, while it is unclear whether chronic low-grade inflammation is a cause or a consequence of impaired cytokine signaling, both can enhance each other, are part of a dysfunctional immune system and probably lead to reduced immune responses that are clinically meaningful, due to their associations with frailty.

Of interest is the association we found in men between higher CRP levels in the previous 20 years, a sign of chronic low grade inflammation, and lower IL-10 induced pSTAT3 responses. Since we also observed lower STAT3 responsiveness to IL-10 in lymphocytes of frailer men, this might suggest that their lymphocytes are less responsive to anti-inflammatory signals and therefore less able to control inflammation. These findings are in agreement with results of previous studies showing that chronically elevated levels of CRP are related to frailty (28–30). Recent studies noted that severely ill COVID-19 patients show elevated levels of IL-10 (31) which led some investigators to propose that IL-10 might contribute to the severity of the disease, because IL-10 is known to have both pro- and anti-inflammatory properties (32). Given that the risk of developing severe symptoms in COVID-19 is greater in older men than in older women (33), and since we showed signs of defective IL-10 signaling in frail men, we speculate that defective downstream IL-10 -STAT3 signaling contributes to the severity of infectious diseases such as COVID-19 due to the reduced immune regulatory function of IL-10. Indeed, JAK-STAT pathway signaling has been shown to be pivotal in developing severe symptoms such as a cytokine release syndrome in COVID-19 patients (34), in particular through STAT3 signaling (35).

Many clinical trials are currently ongoing to investigate efficacy of blocking JAK-STAT signaling with JAK inhibitors in the treatment of COVID-19 (34). While phase II studies showed promising results (36, 37),

early reports from phase III trials are mixed, with some positive (38) and some negative results (39). These mixed results might be explained by different timing of drug administration, and by improper identification of the patients who benefit most from JAK/STAT inhibition (34). This should be addressed in future studies which should also elucidate whether impaired regulatory IL-10 STAT3 signaling can be a marker of developing severe reactions to infectious diseases. This would strengthen the rationale for targeting this pathway. Thus, cellular pSTAT levels may qualify as a biomarker to help identifying patients that benefit most from JAK inhibitor treatment.

An important factor in the association between frailty and reduced cytokine responsiveness could be overweight, since overweight has been associated with impaired JAK-STAT responses in adipocytes (40). We were unable to adjust the results for BMI directly due to the small sample size. However, we did not find associations of pSTAT responses with BMI, which may imply that BMI is not the main driver in the association of pSTAT1, pSTAT3, and pSTAT5 responses in immune cells and frailty.

While we found that cytokine responsiveness is lower in frail people, we did not find an association of frailty with cellular cytokine production after stimulation with TLR agonists such as LPS, R848 and CpG ODN. This might mean that the initial intracellular reaction to pattern associated molecular patterns is still intact, but that the response to TLR-induced cytokines is reduced in frail individuals due to reduced JAK-STAT pathway signaling. Results should be interpreted with care, as the high dosage of the TLR agonists chosen for in vitro stimulation may overcome or mask possible subtle *in-vivo* differences in TLR responses.

To the best of our knowledge, this is the first study describing differences in pSTAT responses of leukocytes in men and women. Lower cellular pSTAT responsiveness in frailer participants was seen in both men and women, with the direction of the correlations being the same in men and women for most of the experimental conditions. However, impaired signaling in frailer participants was seen with different pSTATs in men and women, and in general the observed associations were stronger in men. Of interest is that pSTAT1 cytokine signaling was found to be more impaired in frail women than in men, while frail women were also found to have higher monocyte numbers. This is consistent with impaired phagocyte functioning in frail women, with more monocytes being produced due to their impaired cellular signaling capacity as a possible compensatory mechanism. On the other hand, pSTAT5 signaling was found to be impaired in frail men but not in frail women. It is known that pSTAT5 responses can be initiated by growth hormones (41). Growth hormone secretion is higher in women than in men and is influenced by sex hormones such as estrogens (41), which might explain why we found the associations between pSTAT5 responses and frailty in men but not in women.

Strengths of the study are the in-depth pSTAT signaling assay that we used, which allowed us to characterize a wide range of functional cellular cytokine response signals. Another advantage is that we could build on the extensive information and biomaterials gathered in the course of a unique longitudinal cohort study. This gave us the opportunity to compile and score a comprehensive frailty index in older individuals and to measure inflammatory markers longitudinally over a period of 20 years to quantify

chronic low-grade inflammation. Our study also comes with limitations. First, our sample size was relatively low, so results cannot be extrapolated to the general population. Furthermore, the cellular pSTAT responsiveness could not accurately predict frailty by a prediction model due to the small sample size and the relatively small changes in effect size (data not shown). Another noteworthy point is that, while we used a large panel of cytokines signaling via JAK/STAT pathways we did not study activation of other known STATs, namely STAT2, STAT4 and STAT6. Larger phospho-flow panels may give a more complete description of cellular responsiveness in frail older people. Other innate signaling cytokines such as IL-4 and IL-8 could also be considered in future studies, especially since the function of myeloid lineage cells is thought to become impaired with age (4, 42) and numbers of myeloid cells are higher in frail older people (27). Also, how the results translate to in-vivo signaling remains to be investigated. In-vivo signaling might differ from in-vitro signaling, as the context is different and in vivo cytokine receptor expression might vary due to e.g. the presence of specific antigen, co-stimulation, or a difference in strength and duration of stimulation.

Conclusion

In summary, our study gives important insights into relationships between immune functioning and frailty, and revealed sex-specific differences. We found cellular pSTAT responses to be reduced in older frail individuals. Interestingly, frail men show signs of defective regulatory pSTAT cytokine signaling and this is associated with chronic low-grade inflammation in the past 20 years. The data imply that the JAK/STAT signaling pathway is important in the aging process and therefore markers of this pathway could have utility as biomarkers of frailty. This is of particular interest since multiple drugs have been developed that can target the JAK/STAT pathway. We hope that these data encourage further research investigating how impaired JAK-STAT signaling is related to a poor immunological response to vaccines and infections and to chronic low-grade inflammation in older individuals.

Methods

The study participants take part in the ongoing Doetinchem cohort study (DCS) (25, 26). In the DCS, six consecutive measurement and sampling rounds, every 5 years, have been completed (1993-1997, 1998-2002, 2003-2007, 2008-2012, 2013-2017), while the seventh round is still ongoing (2018-2022). During each measurement round, plasma samples were taken and information regarding the participants' health was collected. The present analysis is based on a sample of 16 men and 18 women who were 65-75 years of age, selected from a subgroup of DCS participants in which we previously analyzed inflammatory marker trajectories (43). The latter were still active in the DCS at least until 2016, had at least 5 plasma samples available from previous rounds, were CMV seronegative at last measurement, and did not use immunosuppressants. From among those, we chose individuals with the highest and with the lowest FI, in equal numbers. The sample size was restricted by budgetary and logistic constraints.

Frailty index

Frailty of participants was evaluated with a frailty index score. Details on the frailty index score used in this study can be found elsewhere (29). In short, the frailty index score was based on previous studies (23, 24, 44, 45) and consists of 36 health deficits assessed by questionnaires and objective measurements. Examples are cognitive deficits obtained from validated cognitive tests, physical deficits such as poor handgrip strength, psychological deficits, and deficits regarding living independently (**Table S2**). The values that the frailty index can take are restricted between 0 (best possible score) and one (worst possible score). The frailty index was calculated based on data of the last two DCS assessment rounds (round 5 and 6) and was validated in the Doetinchem cohort study (29).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Lymphoprep (Progen) density gradient centrifugation, according to the manufacturer's instructions. After isolation, the cells were washed with PBS (Gibco) containing 0.2% FCS, and then frozen in a solution with 90 % fetal calf serum and 10 % dimethyl sulfoxide at -135°C until further use.

PBMC cytokine response analysis using phospho-flow cytometry

PBMCs from all participants (n=34) were stimulated with cytokines to measure JAK-STAT pathway activation by quantifying the phosphorylation of STAT 1, 3 and 5 (Figure 1). We used a barcoding technique as described previously (16, 46). PBMCs were rapidly thawed and washed in RPMI (Thermofisher Scientific) with 10% fetal bovine serum. After 1 hour resting, the PBMCs were stimulated with one cytokine per well (deep well plates, Sigma-Aldrich, 0.5×10^6 PBMCs per well) for 30 minutes at 37°C, 5% CO₂ with the cytokines IL-2 (R&D Systems, 50 ng mL⁻¹), IL-6 (R&D Systems, 25 ng mL⁻¹), IL-10 (Peprotech, 100 ng mL⁻¹), IFN α (R&D Systems, 0.5×10^4 U mL⁻¹) and IFN γ (Peprotech, 50 ng mL⁻¹). One well was reserved as the control condition, without stimulus. Next, PBMCs were fixated (in the deep well plate) with 1.6% paraformaldehyde (Alfa Aesar) for 10 minutes at room temperature, and thereafter permeabilized with ice-cold (-20°C) 100% methanol for five minutes, at 4°C. After permeabilization, the methanol was diluted 1:1 with cold PBS and the individual samples were stained for 30 minutes, 4°C with a combination of Pacific Orange succinimidyl esters (0, 0.13, or 1 μ g mL⁻¹) and Alexa Fluor 750 succinimidyl esters (0, 0.5, or 2 μ g mL⁻¹ Fisher Scientific), to obtain a unique bar code per condition. After barcoding, the PBMCs were extensively washed with PBS/0,5% BSA/2mM EDTA and the PBMCs from the same individual were collected and pooled in one FACS tube (BD Falcon). Thereafter, the PBMCs were stained for 30 minutes at 4°C with CD3(UCHT1)-Pacific Blue, CD4(SK3)-BUV395, CD20(H1)-PerCPCy5.5, and CD33(P67-6)-PECy7 to distinguish CD4⁺ and CD8⁺ T cells, B cells, monocytes, respectively and with the markers STAT1(4a)-Alexa Fluor 488, STAT3(4/P)-Alexa Fluor 647 and STAT5(pY694)-PE to quantify phosphorylation of STATs (**Figure S2**). Samples were measured on a flow cytometer (LSR II Fortessa X20, BD Bioscience).

TLR stimulation of PBMC and cytokine production assay

PBMCs of all participants were thawed, washed and put to rest for 1 hour in RPMI+glutamax with 10% FBS in a 96-wells round-bottom plate (Greiner), at a concentration of 200.000 PBMCs/well. After resting, the PBMCs were stimulated with one stimulus per well for 24 hours at 37°C, 5% CO₂. Stimuli used were LPS EK (E.coli K12 ultrapure) 10 ng mL⁻¹ (TLR4), CpG ODNM362 10 µg mL⁻¹ (TLR9), and R848 10 µg mL⁻¹ (all Invivogen) (TLR7&8). After stimulation, the supernatants were collected and stored at -80°C for later use. The supernatants were thawed and analyzed in two batches on a FACSCanto™ flow cytometer (BD Biosciences), using a custom-made bead-based immunoassay (LEGENDplex™, BioLegend) according to the manufacturer's instructions, for the following cytokines and chemokines: sGP130, IL-6, IFN γ , IL-10, IL-1b, TNF α , MCP-1, RANTES, CXCL8/IL-8, CXCL10/IP10, and IFN α . In addition, concentrations of sIL6R were quantified using a commercially available ELISA kit (R&D Systems).

Plasma inflammatory protein trajectories

To quantify low-grade inflammation, we measured a panel of inflammatory markers in 5 repeated blood samples per individual that were withdrawn at 5-year intervals over the period of approximately 20 years; details are described elsewhere (43). The panel consists of the following cytokines, chemokines and soluble receptors: C-C Motif Chemokine Ligand (CCL) 1/I-309, CCL2/MCP-1, CCL5/RANTES, CCL11/Eotaxin, CCL27/C-TACK, C-X-C Motif Chemokine Ligand (CXCL) 9 /MIG, CXCL10/IP-10, CXCL11/I-TAC, IL-10, IL-6, soluble CD40 ligand (sCD40L), soluble CD14 (sCD14), soluble IL-6 receptor (sIL-6R), glycoprotein 130 (GP130), Complement 5a (C5a), Brain-derived neurotrophic factor (BDNF), and soluble P-selectin. Plasma levels were measured in a multiplex immunoassay (Luminex core facility lab, UMC medical center, Utrecht, The Netherlands). Samples were stored at -80⁰ Celsius and only thawed on the morning of the measurement, with all samples from the same participant measured on the same plate. Levels of C-reactive protein (CRP) had been measured earlier in separate plasma samples and in a separate assay (47).

Statistical analysis

Association studies

We used the permutation version of the Spearman's test or, when appropriate, Wilcoxon's test as implemented in the *coin* R package (48) to test associations for all our research questions. These association studies were performed separately for men and women. Associations were adjusted for possible confounding by batch effects. For all these association studies, we accounted for multiple testing by controlling the false discovery rate (49). A cutoff of the false discovery rate of 15% was chosen based on the exploratory nature of this research. This means that, in theory, of all findings reported here at most 15% could be false positives. All details regarding the outcomes of the association studies, including p-values, are shown in supplementary information (**Table S2,S4,S5,S6,S7,S8,S9, and S10**).

All analyses were performed in R (version **3.6.2**) (50). Several packages were used for general data wrangling (51, 52), data visualization (53–55), and customizing tables (56,57). Clusters of inflammatory markers within a heatmap were defined with a hierarchical cluster algorithm using the complete linkage

criterion. The clustering was performed on the dissimilarity matrix which was obtained from data of the ρ values.

Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the University Medical Center in Utrecht, The Netherlands. The participants gave written informed consent for every DCS round and for this subcohort study separately

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to privacy and ethical restrictions but are available from the corresponding author on reasonable request.

Competing interest

None to declare.

Funding

The Doetinchem Cohort Study is funded by the Dutch Ministry of Health, Welfare and Sport. Additional funding for the current study was also provided by the Ministry. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author's contributions

SP and MV contributed to the development and maintenance of the Doetinchem Cohort Study. LS, PE, AB, and MB developed and implemented the DCS subcohort study design. MZ and LS developed the flow cytometry laboratory protocols and MZ performed the experimental work in the laboratory and performed initial data cleaning. JF provided the initial data analysis framework, and LS and PE implemented it in the manuscript. LS performed data cleaning, wrote the initial draft of the manuscript, and provided visualizations. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank the DCS respondents for their participation in the study and the epidemiologists and fieldworkers of the Municipal Health Service in Doetinchem for their contribution to the data collection. In addition, we would like to thank Petra Vissink for her help with the blood sample collection. Figure 1 was created using BioRender.com.

References

1. Kennedy BK, Berger SL, Brunet A, Campisi J, Cuervo AM, Epel ES, et al. Geroscience: Linking Aging to Chronic Disease. *Cell*. 2014 Nov 6;159(4):709–13.
2. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*. 2006;24.
3. Gustafson CE, Kim C, Weyand CM, Goronzy JJ. Influence of immune aging on vaccine responses. *Journal of Allergy Clinical Immunology*. 2020 May;145(5):1309–21.
4. Aiello A, Farzaneh F, Candore G, Caruso C, Davinelli S, Gambino CM, et al. Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically? A Review of Potential Options for Therapeutic Intervention. *Front Immunol*. 2019 Sep;25:10:2247.
5. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Annals of the New York Academy of Sciences*. 2006 Jan 25;908(1):244–54.
6. Fulop T, Witkowski JM, Olivieri F, Larbi A. The integration of inflammaging in age-related diseases. *Seminars in Immunology*. 2018 Dec 1;40:17–35.
7. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, et al. Chronic inflammation in the etiology of disease across the life span. *Nat Med*. 2019 Dec;25(12):1822–32.
8. Franceschi C, Garagnani P, Vitale G, Capri M, Salvioli S. Inflammaging and ‘Garb-aging.’ *Trends in Endocr Metab*. 2017 Mar;28(3):199–212.
9. van Deursen JM. The role of senescent cells in ageing. *Nature*. 2014 May 1;509(7501):439–46.
10. Villarino AV, Kanno Y, O’Shea JJ. Mechanisms and consequences of Jak–STAT signaling in the immune system. *Nat Immunol*. 2017 Apr;18(4):374–84.
11. O’Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annual review of medicine*. 2015;66:311–28.
12. Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, et al. Impaired response to interferon- α/β and lethal viral disease in human STAT1 deficiency. *Nat Genet*. 2003 Mar;33(3):388–91.
13. Sancho-Shimizu V, Perez de Diego R, Jouanguy E, Zhang S-Y, Casanova J-L. Inborn errors of anti-viral interferon immunity in humans. *Current Opinion in Virology*. 2011 Dec;1(6)(1):487–96.

14. Sandborn WJ, Su C, Sands BE, D'Haens GR, Vermeire S, Schreiber S, et al. Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *New England Journal of Medicine*. 2017 May 4;376(18):1723–36.
15. Heijde D van der, Strand V, Tanaka Y, Keystone E, Kremer J, Zerbini CAF, et al. Tofacitinib in Combination With Methotrexate in Patients With Rheumatoid Arthritis: Clinical Efficacy, Radiographic, and Safety Outcomes From a Twenty-Four–Month, Phase III Study. *Arthritis Rheumatology*. 2019;71(6):878–91.
16. Shen-Orr SS, Furman D, Kidd BA, Hadad F, Lovelace P, Huang YW, et al. Defective Signaling in the JAK-STAT Pathway Tracks with Chronic Inflammation and Cardiovascular Risk in Aging Humans. *Cell Syst*. 2016 Oct 26;3(4):374-384 e4.
17. Piber D, Olmstead R, Cho JH-J, Witarama T, Perez C, Dietz N, et al. Inflammaging: Age and Systemic, Cellular, and Nuclear Inflammatory Biology in Older Adults. Le Couteur D, editor. *The Journals of Gerontology: Series A*. 2019 Oct 4;74(11):1716–24.
18. Febvre-James M, Lecureur V, Fardel O. Potent repression of C-reactive protein (CRP) expression by the JAK1/2 inhibitor ruxolitinib in inflammatory human hepatocytes. *Inflamm Res*. 2020 Jan 1;69(1):51–62.
19. Xu M, Tchkonja T, Ding H, Ogrodnik M, Lubbers ER, Pirtskhalava T, et al. JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. *Proc Natl Acad Sci USA*. 2015 Nov 17;112(46):E6301–10.
20. Bailey KL, Smith LM, Heires AJ, Katafiasz DM, Romberger DJ, LeVan TD. Aging leads to dysfunctional innate immune responses to TLR2 and TLR4 agonists. *Aging Clin Exp Res*. 2019 Sep;31(9):1185–93.
21. van den Biggelaar AHJ, Huizinga TWJ, de Craen AJM, Gussekloo J, Heijmans BT, Frölich M, et al. Impaired innate immunity predicts frailty in old age. The Leiden 85-plus study. *Experimental Gerontology*. 2004 Sep 1;39(9):1407–14.
22. Wang Q, Westra J, van der Geest KSM, Moser J, Bijzet J, Kuiper T, et al. Reduced levels of cytosolic DNA sensor AIM2 are associated with impaired cytokine responses in healthy elderly. *Exp Gerontol*. 2016 Jun;78:39–46.
23. Mitnitski AB, Mogilner AJ, Rockwood K. Accumulation of deficits as a proxy measure of aging. *TheScientificWorldJournal*. 2001 Aug 8;1:323–36.
24. Searle SD, Mitnitski A, Gahbauer EA, Gill TM, Rockwood K. A standard procedure for creating a frailty index. *BMC Geriatr*. 2008 Sep 30;8:24.
25. Picavet HSJ, Blokstra A, Spijkerman AMW, Verschuren WMM. Cohort Profile Update: The Doetinchem Cohort Study 1987-2017: lifestyle, health and chronic diseases in a life course and ageing perspective. *Int J Epidemiol*. 2017 Dec 1;46(6):1751–1751g.
26. Verschuren WM, Blokstra A, Picavet HS, Smit HA. Cohort profile: the Doetinchem Cohort Study. *Int J Epidemiol*. 2008 Dec;37(6):1236–41.

27. Samson LD, Boots AMH, Ferreira JA, Picavet HSJ, de Rond LGH, de Zeeuw-Brouwer M, et al. In-depth immune cellular profiling reveals sex-specific associations with frailty. *Immun Ageing*. 2020 Jun;23(1):20. 17(.
28. Marcos-Pérez D, Sánchez-Flores M, Maseda A, Lorenzo-López L, Millán-Calenti JC, Gostner JM, et al. Frailty in Older Adults Is Associated With Plasma Concentrations of Inflammatory Mediators but Not With Lymphocyte Subpopulations. *Front Immunol*. 2018 May;16:9:1056.
29. Samson LD, Boots AMH, Verschuren WMM, Picavet HSJ, Engelfriet P, Buisman A-M. Frailty is associated with elevated CRP trajectories and higher numbers of neutrophils and monocytes. *Exp Gerontol*. 2019;125:110674.
30. Walker KA, Walston J, Gottesman RF, Kucharska-Newton A, Palta P, Windham BG. Midlife Systemic Inflammation is Associated with Frailty in Later Life: The ARIC Study. *The journals of gerontology Series A, Biological sciences and medical sciences*. 2018 Mar 9;gly045–gly045.
31. Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. *Emerging Microbes & Infections*. 2020 Jan 1;9(1):1123–30.
32. Lu L, Zhang H, Dauphars DJ, He Y-W. A Potential Role of Interleukin 10 in COVID-19 Pathogenesis. *Trends in Immunology [Internet]*. 2020 Nov 2 [cited 2020 Dec 22]; Available from: <http://www.sciencedirect.com/science/article/pii/S1471490620302568>.
33. Takahashi T, Ellingson MK, Wong P, Israelow B, Lucas C, Klein J, et al. Sex differences in immune responses that underlie COVID-19 disease outcomes. *Nature*. 2020 Dec;588(7837):315–20.
34. Luo W, Li Y-X, Jiang L-J, Chen Q, Wang T, Ye D-W Targeting JAK-STAT Signaling to Control Cytokine Release Syndrome in COVID-19. *Trends in Pharmacological Sciences*. 2020 Aug 1;41(8):531–43.
35. Hojyo S, Uchida M, Tanaka K, Hasebe R, Tanaka Y, Murakami M, et al. How COVID-19 induces cytokine storm with high mortality. *Inflammation and Regeneration*. 2020 Oct 1;40(1):37.
36. Gozzetti A, Capochiani E, Bocchia M. The Janus kinase 1/2 inhibitor ruxolitinib in COVID-19. *Leukemia*. 2020 Oct;34(10):2815–6.
37. La Rosée F, Bremer HC, Gehrke I, Kehr A, Hochhaus A, Birndt S, et al. The Janus kinase 1/2 inhibitor ruxolitinib in COVID-19 with severe systemic hyperinflammation. *Leukemia*. 2020 Jul;34(7):1805–15.
38. Kalil AC, Patterson TF, Mehta AK, Tomashek KM, Wolfe CR, Ghazaryan V, et al. Baricitinib plus Remdesivir for Hospitalized Adults with Covid-19. *New England Journal of Medicine [Internet]*. 2020 Dec 11 [cited 2020 Dec 22]; Available from: <https://www.nejm.org/doi/10.1056/NEJMoa2031994>.
39. Novartis. Novartis provides update on RUXCOVID study of ruxolitinib for hospitalized patients with COVID-19 [Internet]. N. 2020 [cited 2020 Dec 23]. Available from: <https://www.novartis.com/news/media-releases/novartis-provides-update-ruxcovid-study-ruxolitinib-hospitalized-patients-covid-19>.
40. Dodington DW, Desai HR, Woo M. JAK/STAT – Emerging Players in Metabolism. *Trends in Endocrinology & Metabolism*. 2018 Jan 1;29(1):55–65.

41. Sehgal PB, Yang Y-M, Yuan H, Miller EJ. STAT5a/b contribute to sex bias in vascular disease: A neuroendocrine perspective. *JAK-STAT*. 2015 Jul 3;4(3):1–20.
42. Duong L, Radley H, Lee B, Dye D, Pixley F, Grounds M, et al. Macrophage function in the elderly and impact on injury repair and cancer. *Immunity Ageing*. 2021 Jan;13(1):4. 18(.
43. Samson LD, Buisman A-M, Ferreira JA, Picavet HSJ, Verschuren WMM, Boots AMH, et al. Relation of inflammatory marker trajectories with frailty and aging in a 20-year longitudinal study. *bioRxiv*. 2021 Feb 16;2021.02.10.430670.
44. Collerton J, Martin-Ruiz C, Davies K, Hilkens CM, Isaacs J, Kolenda C, et al. Frailty and the role of inflammation, immunosenescence and cellular ageing in the very old: cross-sectional findings from the Newcastle 85+ Study. *Mechanisms of ageing and development*. 2012 Jun;133(6):456–66.
45. Schoufour JD, Erler NS, Jaspers L, Kiefte-de Jong JC, Voortman T, Ziere G, et al. Design of a frailty index among community living middle-aged and older people: The Rotterdam study. *Maturitas*. 2017 Mar;97:14–20.
46. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods*. 2006 May;3(5):361–8.
47. Hulsegge G, Herber-Gast GC, Spijkerman AM, Susan H, Picavet J, van der Schouw YT, et al. Obesity and Age-Related Changes in Markers of Oxidative Stress and Inflammation Across Four Generations. *Obesity (Silver Spring)*. 2016 Jun;24(6):1389–96.
48. Hothorn T, Winell H, Hornik K, de Wiel MA van, Zeileis A coin: Conditional inference procedures in a permutation test framework [Internet]. 2019. Available from: <https://CRAN.R-project.org/package=coin>.
49. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)*. 1995;57(1):289–300.
50. R Core Team. R: A language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2019. Available from: <https://www.R-project.org/>.
51. Wickham H, François R, Henry L, Müller K. dplyr: A grammar of data manipulation [Internet]. 2020. Available from: <https://CRAN.R-project.org/package=dplyr>.
52. Wickham H, Henry L. tidyr: Tidy messy data [Internet]. 2020. Available from: <https://CRAN.R-project.org/package=tidyr>.
53. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*. 2016 Sep 15;32(18):2847–9.
54. Wickham H, Chang W, Henry L, Pedersen TL, Takahashi K, Wilke C, et al. ggplot2: Create elegant data visualisations using the grammar of graphics [Internet]. 2019. Available from: <https://CRAN.R-project.org/package=ggplot2>.
55. Wilke CO. cowplot: Streamlined plot theme and plot annotations for “ggplot2” [Internet]. 2019. Available from: <https://CRAN.R-project.org/package=cowplot>.

56. Xie Y. knitr: A general-purpose package for dynamic report generation in r [Internet]. 2020. Available from: <https://yihui.org/knitr/>.
57. Zhu H. kableExtra: Construct complex table with “kable” and pipe syntax [Internet]. 2020. Available from: <https://CRAN.R-project.org/package=kableExtra>.

Figures

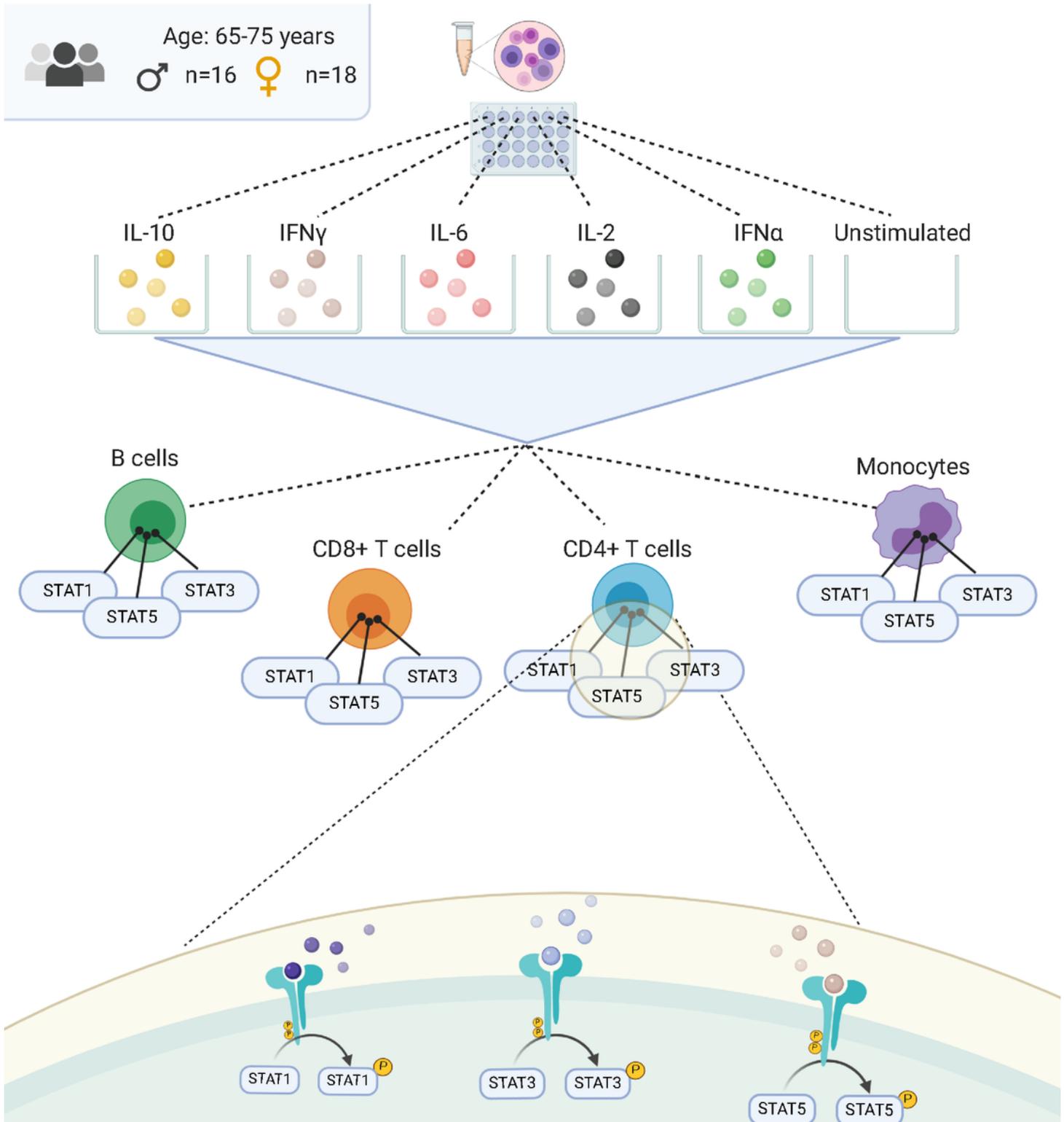


Figure 1

Study and experimental design. CMV-seronegative participants were selected from the Doetinchem cohort study. PBMCs were thawed and stimulated with the indicated cytokines or were left unstimulated (baseline). Intranuclear immunofluorescence staining was performed to quantify phosphorylation of STAT1, 3, and 5 proteins, in combination with surface staining for lineage-specific markers to distinguish between B cells, monocytes, and T cells (CD8+ and CD4+).

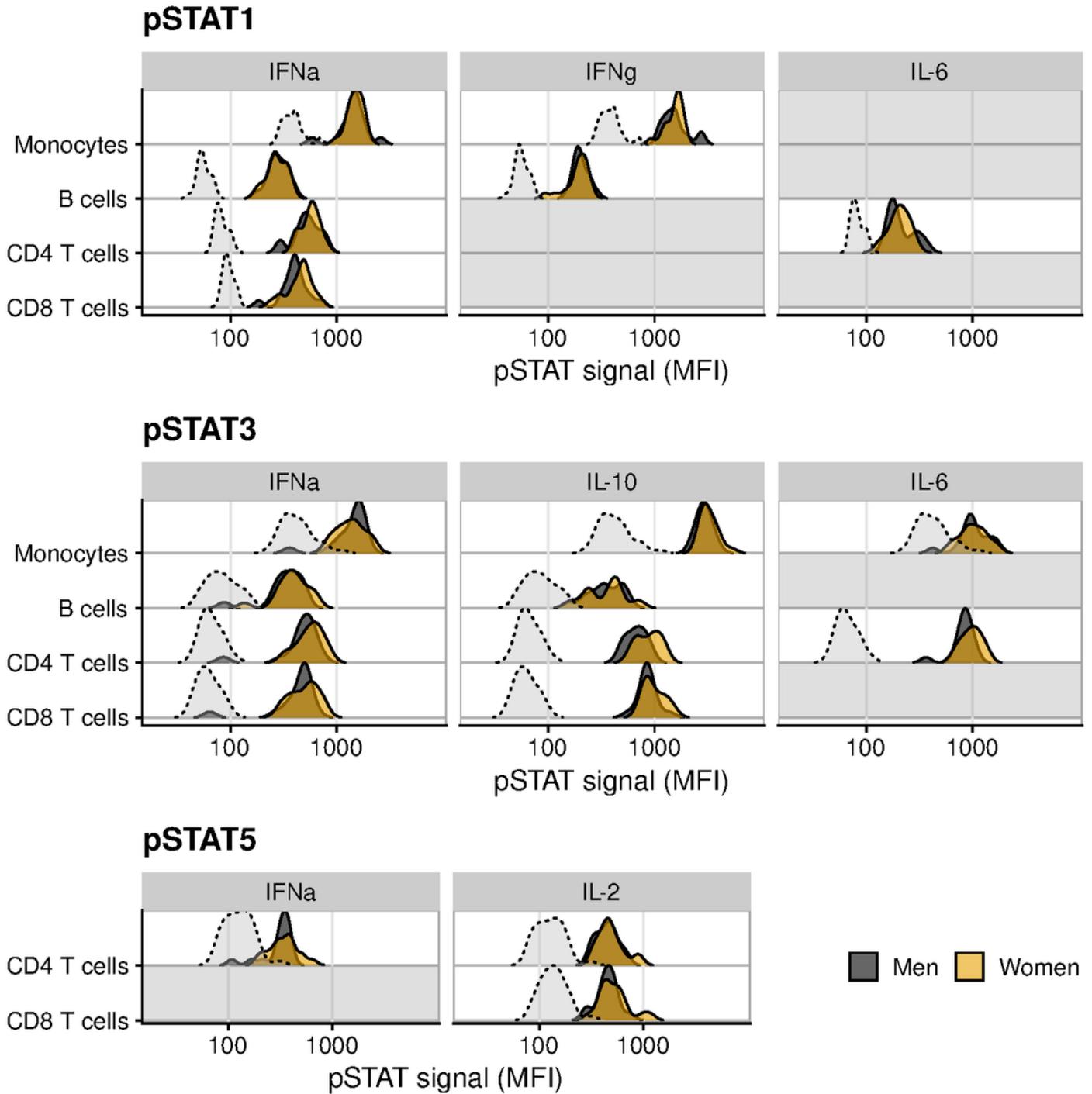


Figure 2

Levels of phosphorylated STAT proteins (pSTAT1, pSTAT3, and pSTAT5), measured in CD4+ T cells, CD8+ T cells, B cells, and monocytes. Grey surface area with dashed outline shows baseline (unstimulated) pSTAT levels. Dark grey and orange surface areas with solid outline show pSTAT immune cell responses to stimulation in men and women, respectively. Shown are 20 out of 60 experimental stimuli conditions that induced a robust change (fold change >2) compared to the baseline condition in CD4+, CD8+, B cells and monocytes.

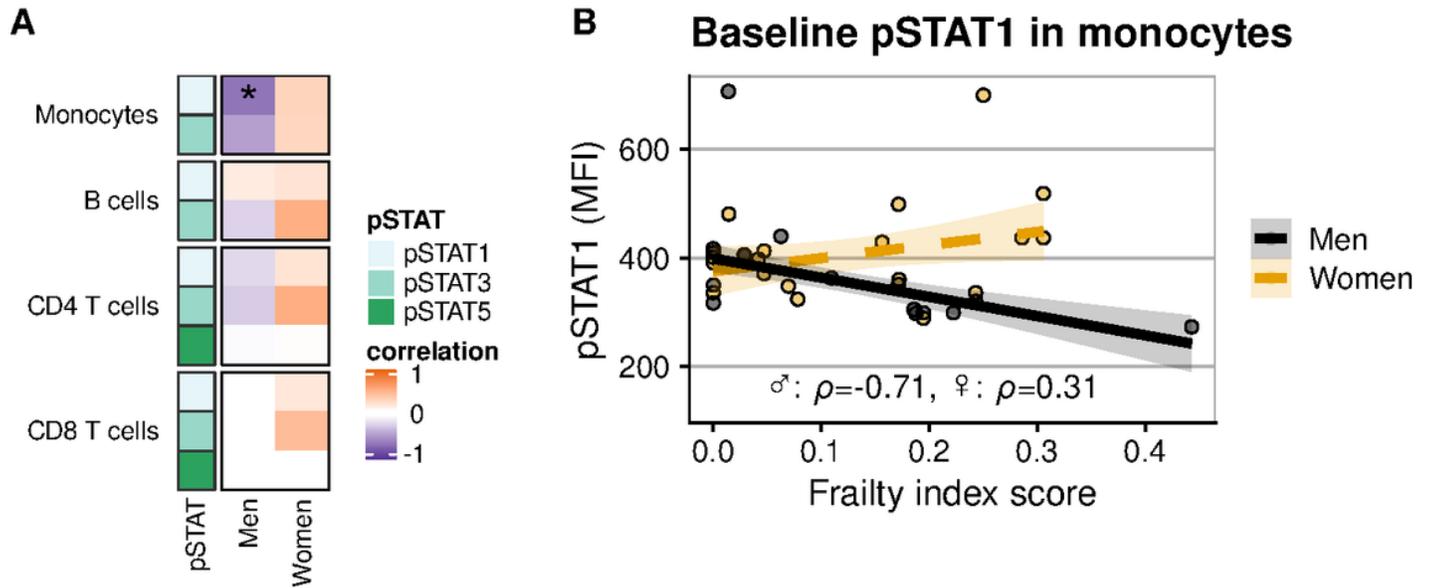


Figure 3

(A) Heatmap showing correlation coefficients of the relation between frailty and the baseline (unstimulated) phosphorylation of STAT1, STAT3, and STAT5 in monocytes, B cells, and CD4+ and CD8+ T cells. Every box displays the Spearman's ρ value based on data of 16 men and 18 women. (B) Baseline (unstimulated) pSTAT1 expression in monocytes. Solid trendline in (B) indicates that an association was found.

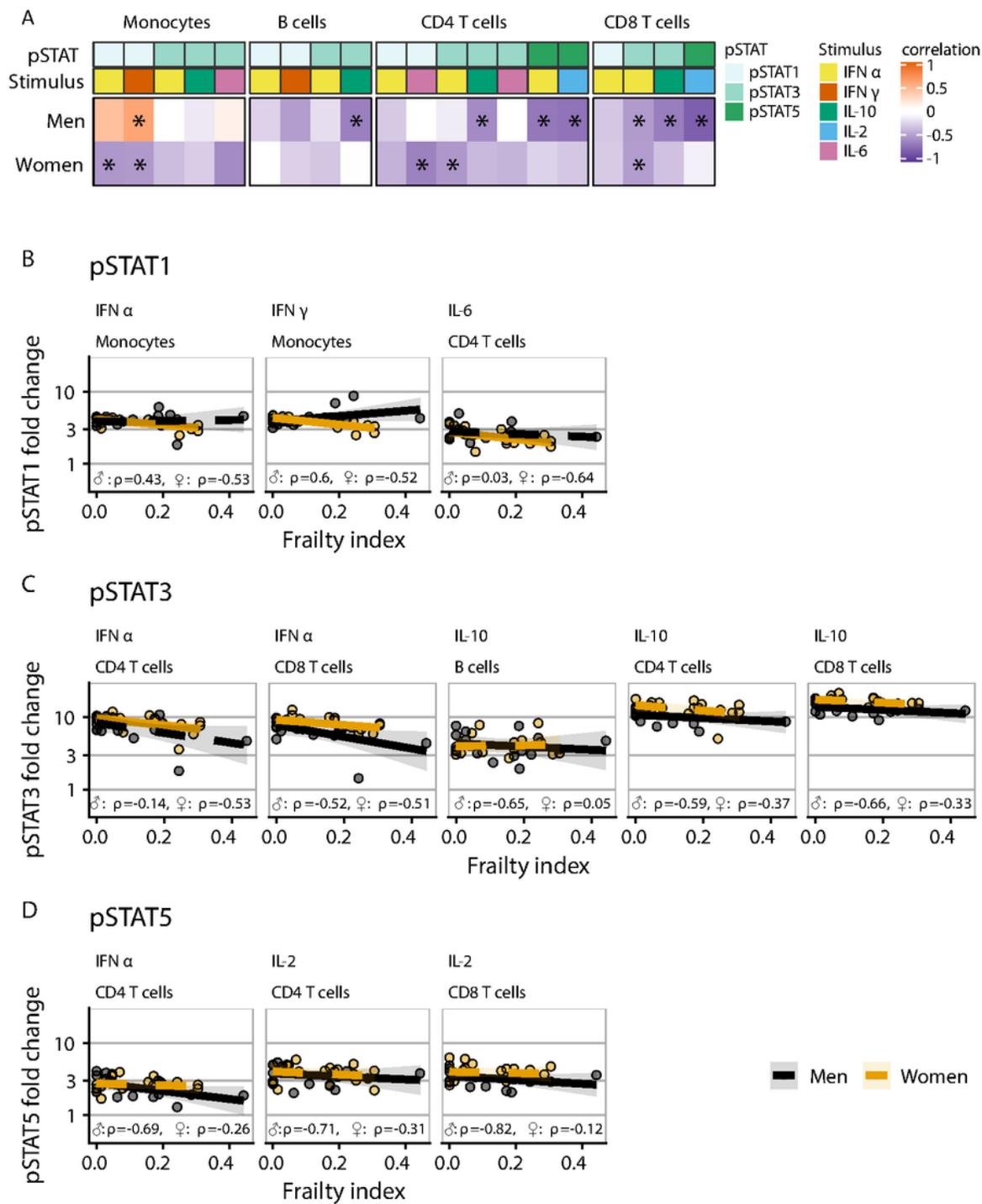


Figure 4

(A) heatmap showing relations between frailty and the cellular response to cytokines detected by phosphorylation of STAT1, STAT3, and STAT5 (fold change from baseline levels) in monocytes, B cells, CD4+ T cells and CD8+ T cells. Every box displays the Spearman's ρ value, based on data of 16 men and 18 women. Scatterplots of conditions in which an association was detected for pSTAT1 (B), pSTAT3 (C) and pSTAT5 responses. For comparison, data of men and women are shown in the same plots. Solid

trendlines indicate that an association was found, and dashed line indicates that no association was found.

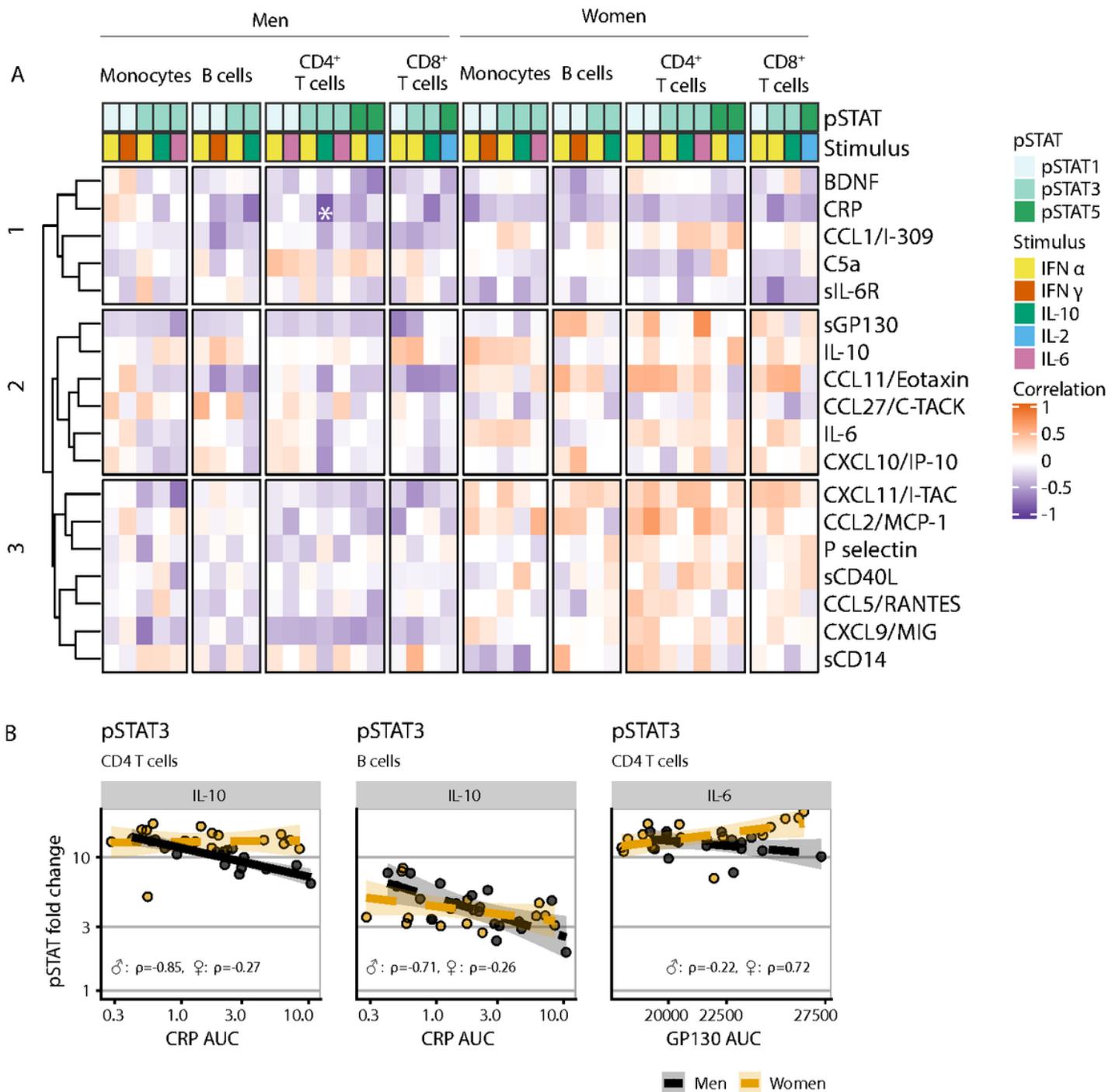


Figure 5

(A) heatmap showing Spearman's correlation coefficients between immune cell pSTAT responses and systemic levels of inflammatory markers measured over the last 20 years (AUC). The magnitude and direction of the correlation are indicated by shades of color. Every correlation is based on n=18 women or n=16 men. A star indicates that an association was found. (B) Scatterplots showing relationships

between immune cell pSTAT responses and systemic levels of inflammatory markers (AUC), of which Spearman's ρ was >0.7 or <-0.7 . For comparison, data of men and women are shown in the same plots. Solid trendlines indicate that an association was found, and dashed line indicates that no association was found.

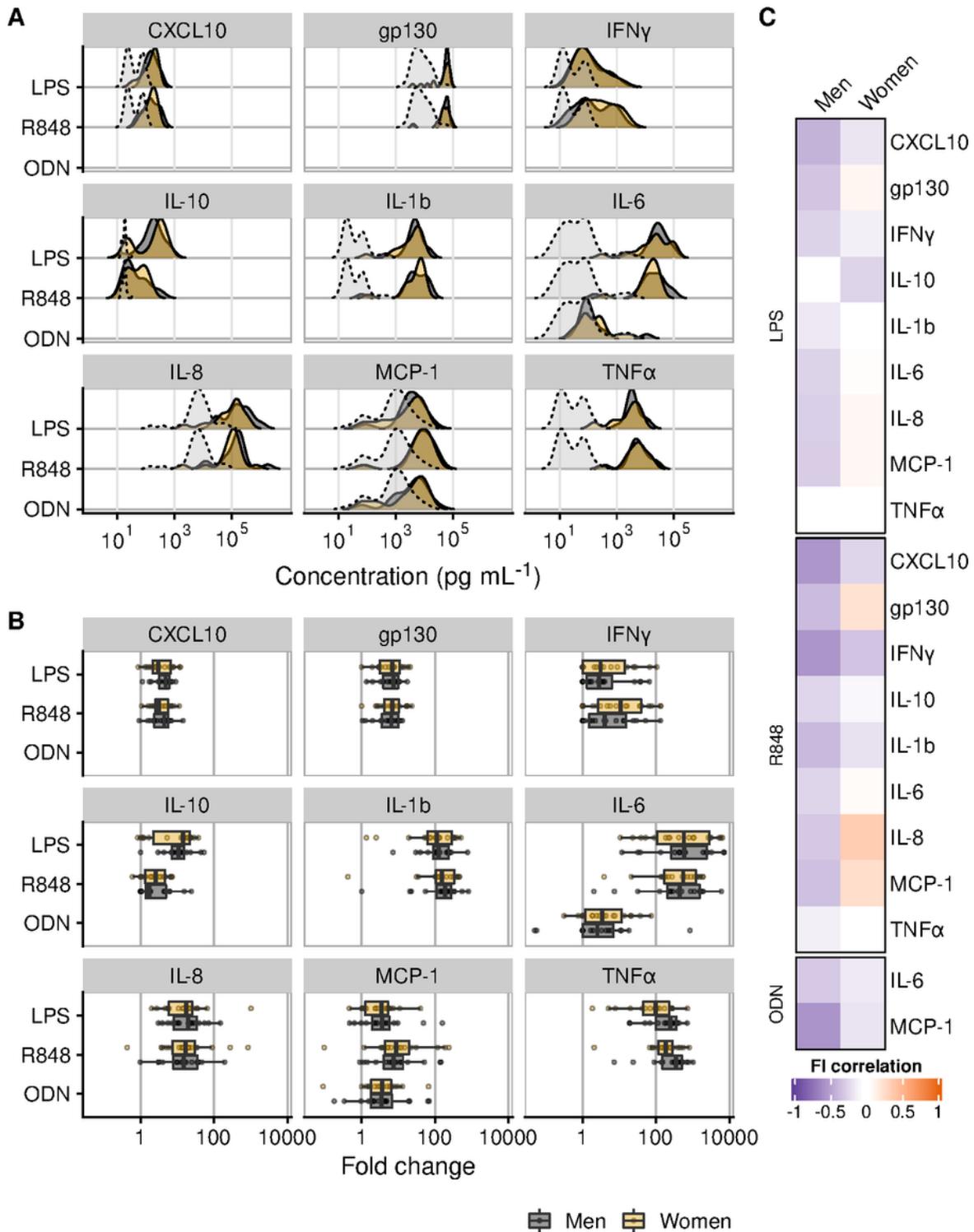


Figure 6

(A) Density plots of cytokine production by PBMCs after 24h stimulation with medium (light grey density, spontaneous cytokine production) or with R848, ODN, or LPS. (B) Cytokine production by PBMCs upon 24h stimulation with TLR4 (LPS), TLR7/8 (R848), and TLR9 (CpG ODN) agonists. Plots are shown only for cytokines with a fold change > 2. (C) Heatmap showing Spearman's ρ values of correlations between frailty and PBMC cytokine production.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SamsonetalpSTATfrailtysupplementarymaterial.pdf](#)