In-depth immune cellular profiling reveals sex-specific associations with frailty

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Research

Keywords: frailty, immune cellular profiling, immunosenescence, immune homeostasis, healthy aging

Posted Date: March 10th, 2020

DOI: https://doi.org/10.21203/rs.2.19660/v2

Version of Record: A version of this preprint was published on June 23rd, 2020. See the published version at https://doi.org/10.1186/s12979-020-00191-z.

RESEARCH

In-depth immune cellular profiling reveals sex-specific associations with frailty

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Abstract

Background: With advancing age, the composition of leukocyte subpopulations in peripheral blood is known to change, but how this change differs between men and women and how it relates to frailty is poorly understood. Thus, our aim in this exploratory study was to investigate whether frailty is associated with changes in immune cell subpopulations and whether associations differ between men and women. Therefore, we performed in-depth immune cell phenotyping by enumerating a total of 37 subsets of T cells, B cells, NK cells, monocytes, and neutrophils in peripheral blood of 289 elderly people between 60-87 years of age. Associations between frailty and each immune cell subpopulation were tested separately in men and women and were adjusted for age and CMV serostatus. In addition, a random forest algorithm was used to predict a participant's frailty score based on enumeration of immune cell subpopulations.

Results: In an association study, frailty was observed to be associated with increases in numbers of neutrophils in both men and in women. Furthermore, sex-specific associations were found. Frailer women, but not men, showed higher numbers of total and $CD16^-$ monocytes and lower numbers of $CD56^+$ T cells. Overall, the accuracy of the predictions in the random forest analysis was low (9.2% explained variance in men and 12.2% in women). Yet, the random forest analysis confirmed all associations mentioned above, but did not confirm a possible negative association between frailty and late differentiated $CD4^+$ TemRA cells in women. Moreover, the random forest analysis revealed additional relationships with frailty, with frailer men showing higher $CD16^+$ monocytes and lower naïve B cell numbers. Other important variables for predicting frailty were plasmablast numbers in men and total T cell numbers in women.

Conclusions: We report on observed associations of frailty with elevated myeloid cell numbers in men and women. In-depth immune cellular profiling also revealed sex-specific associations of frailty with several immune subpopulations. However, an expected positive association between frailty and memory T cells was not observed. We hope that our study will prompt further investigation into the immune mechanisms associated with the development of frailty in men and women.

Keywords: frailty; immune cellular profiling; immunosenescence; immune homeostasis; healthy aging

1 Background

While aging is inevitable, some people remain healthy until an advanced age while others suffer from age-related diseases early in life. The reasons for these vastly different aging patterns are still poorly understood. To protect the body from damage normally associated with aging, a balanced immune system is needed [1], with different subsets of immune cells working in close harmony. One telling sign of a disturbed immune balance is a state of chronic low-grade inflammation, which may be revealed by measuring biomarkers such as C-reactive protein [1, 2, 3]. It remains a challenge, however, to identify other biomarkers of the immune system that signal or explain differences in aging patterns.

Both innate and adaptive immune cell lineages are essential for a proper functioning of the immune system. It is well-established that the composition of the immune cell repertoire—that is the relative and absolute abundances of the various subsets of immune cells—changes with age [4]. However, it is largely unknown how changes in the immune cellular composition differ in individuals who age in good health from those who become frail at a relatively early age. Several studies have been performed on this subject, but some of these were done in the extreme elderly of 85 years old or above [5], while others focused on a limited set of lymphocyte phenotypes[6, 7, 8]. Importantly, few studies have been performed enumerating a comprehensive set of immune phenotypes in freshly drawn whole blood samples.

Recently, we investigated associations between frailty and absolute numbers of the major immune cell subsets in fresh whole blood. In that study, we detected associations between frailty and numbers of granulocytes and monocytes [3]. However, given the complex nature of the immune system and its specialized functions, that are mediated by various immune cell subpopulations, it is to be expected that changes in major immune cell subsets are accompanied by more subtle, yet potentially important, shifts in smaller subpopulations that are functionally distinct. This hypothesis prompted us to extend our previous study with an in-depth analysis of frailty in relation to various immune cell subpopulations.

Since men and women tend to age differently [9], we deemed it important to consider differences between the sexes. Furthermore, cytomegalovirus (CMV) serostatus should be taken into account, because chronic CMV infection is well known to impact numbers of immune cell subpopulations [10, 11]. The exploratory approach taken in this study involves the investigation of a large number of potential relations between variables. Moreover, given the heterogeneity of our study population, these variables are expected to show much variability. Due care should thus be taken in choosing appropriate methods of analysis that keep the risk of finding spurious associations within bounds, while at the same time allowing the detection of real associations. Therefore, we analyzed the data using robust, "non-parametric", methods of analysis, as further described below.

Thus, our main goal in this study was to explore how general health, as expressed in terms of a frailty index, is associated with an extensive set of immune cell subpopulations in peripheral blood of older adults. Our secondary goal was to explore how these immune cell subpopulations differ between the sexes or according

to CMV serostatus. In order to do so, we employed extensive immune profiling, enumerating 37 immune subsets and describing the expression of several surface markers on immune cells in fresh whole blood samples from 289 older people aged between 60-87 years that were selected from participants in an ongoing cohort study in the Netherlands [12, 13].

2 Methods

2.1 DCS subcohort selection

A subcohort was selected from the Doetinchem cohort study (DCS) [12, 13]. Details on this subcohort and its selection have been described previously [3]. Briefly, the study involved 289 active DCS participants, aged 60-87 years, who were selected as an age- and sex- stratified sample, with selection of equal numbers of the healthiest, intermediate, and frailest participants. By healthiest/frailest we mean those belonging to the 15% individuals with the lowest/highest frailty index score (see below for the definition of frailty index) compared to their age- and sex-matched peers. By intermediate we mean the remaining 70% of the DCS participants.

2.2 Frailty index

A frailty index was constructed based on previous studies [5, 14, 15, 16] and was adapted to the data available in the DCS; it was recently validated within the DCS by [3]. This frailty index incorporates 36 possible "health deficits" such as presence of chronic disease or reduced physical functioning. The values of the frailty index are restricted to lie between zero and one, zero representing the 'best' (0 out of 36 deficits present) and one representing the 'worst' (all 36 deficits present) health status. Since it has 36 categories, this frailty index is by approximation a continuous variable. Using the index, a frailty 'score' was calculated for each individual, based on data collected during the DCS assessment round 6 (2012-2017). Twelve out of 289 individuals who had participated in our DCS subcohort, had not participated in DCS assessment round 6 and thus, their frailty index score was missing.

2.3 Whole blood lymphocyte phenotyping

Fresh whole blood samples from the DCS subcohort participants were collected between August 2016 and March 2017 and were processed and analyzed within 6 hours on a 4-laser LSRII Fortessa X20 flow cytometer (BD Biosciences) for absolute numbers of leukocyte subsets (cell counts μL^{-1}). Two labeled antibody panels per participant were used with a lyse-no-wash protocol, one in a TruCOUNT® tube (BD Biosciences) and one in a common Falcon tube. In both panels we used the fluorochrome-conjugated antibodies CD3(UCHT1)-BV711 (BD) and CD27(M-T271)-BV421 (Biolegend). In the TruCOUNT® tube, we additionally used CD56(B159)-APC, CD8(SK1)-FITC, CD16(B73.1)-PE, CD4(SK3)-PerCPCy5.5, IgD(ia6-2)-BB515, CD38(HB7)-APC-H7, HLA-DR(G46.6)-PECF594 (all BD Biosciences), CD19(J3-119)-PECy7 (Beckman Coulter), and CD45(GA90)-OC515 (Cytognos). In the second tube we additionally used the following fluorochrome-conjugated antibodies: CD127(hIL-7R-M21)-PE, CD25(2A3)-BB515, CCR7(150503)-PECF594, CD28(CD28.2)-PerCPCy5.5, CD8(SK1)-APC-H7 (all BD Biosciences), CD4(RPA-T4)-BV510, CD45RA(HI100)-BV650 (all Biolegend), and CXCR5(51505)-APC (R&D Systems). Absolute cell numbers in the Falcon tube were calculated

by using the CD3 T cell ratio between both tubes and the bead count in the TruCOUNT® tube.

For phenotype definitions and gating strategies, see Table S1 and Figures S1, S2, and S3. Neutrophils were gated as CD45 and SSC^{BRIGHT} and $CD45^{DIM}$ and were additionally analyzed not only regarding cell numbers but also with respect to CD16 expression. CD16 is usually expressed on the surface of neutrophils [17] and is seen as a neutrophil maturation marker [18]. Lower expression of CD16 by neutrophils was seen in several diseases and in states of neutropenia [19]. Monocytes were gated as $SSC^{DIM}CD45^{DIM}$ and, to ensure that B cells or T cells did not contaminate the gate, as $CD3^{-}CD19^{-}$. Monocytes were further sub-classified into non-classical and classical monocytes based on CD16 expression, and were additionally analyzed based on the expression of HLA-DR and CD38, since HLA-DR expression on monocytes is thought to be lower [20] and CD38 expression higher [21] in inflammatory conditions. NK cells were gated and subdivided based on their CD16 and CD56 expression [22]. For memory T cells and regulatory T cells, gating was done as described previously [23, 24, 25] and was performed similarly in both $CD4^{+}$ and $CD8^{+}$ T cells. In short, $CCR7^{+}$ $CD4^{+}/CD8^{+}$ T cells were classified as either naïve ($CD45RA^{+}CCR7^{+}$) or central memory ($CCR7^{+}CD45RA^{-}$) T cells. $CCR7^{-}$ $CD4^{+}/CD8^{+}$ T cells were divided into effector memory T cells (Tem, $CCR7^{-}CD45RA^{-}$) and effector memory T cells re-expressing CD45RA (TemRA, $CCR7^{-}CD45RA^{+}$) T cells. Finally, these T cells were further subclassified into early ($CD27^{+}CD28^{+}$) and late stage ($CD27^{-}CD28^{-}$) Tem or TemRA cells. The B cell subsets were defined by means of CD19, CD27, and CD38 expression [24], with naïve B cells defined as $CD19^{+}CD38^{DIM}CD27^{-}$ and memory B cells as $CD19^{+}CD38^{DIM}CD27^{+}$ (Figure S3 and Table S1). As an additional analysis, we also calculated proportions of the immune cell subpopulations. Proportions were expressed as the percentage of their major cell lineage (T cells, B cells, NK cells, or monocytes) or, for all $CD4^{+}$ and $CD8^{+}$ subpopulations, as the percentage of $CD4^{+}$ and $CD8^{+}$ T cells, respectively. Some subpopulations were not clearly distinguishable from debris and the corresponding data were excluded from analysis. This occurred for the general lymphocyte subset in four participants, for the monocyte subsets in three participants, and for both the lymphocyte and monocyte subsets in three participants. Unwanted fluorochrome excitation spillover was automatically corrected for by using BDComp beads (BD Biosciences). Gating of cellular subsets was performed in FlowJo V10 (FlowJo company).

2.4 Anti-cytomegalovirus antibodies

IgG antibodies against CMV were quantified using a multiplex immunoassay (MIA) that was developed in-house and was based on a commercially available Cytomegalovirus IgG ELISA kit (EUROIMMUN, Germany) [26]. A cutoff for CMV-seropositivity was calculated by first pooling IgG CMV antibody concentrations of people from multiple cohorts [27, 3, 28, 29], with all concentrations measured using the same in-house developed assay (n=1415, age range: 4-89 years). With these pooled concentrations, we used mixture modeling [30] to define two distributions, representing respectively CMV-seronegative and CMV-seropositive individuals. The CMV-seropositivity cutoff was defined as the intersection between the two distributions, as described previously for varicella zoster virus [31].

2.5 Statistical methods

As this was an exploratory study investigating a large number of variables and their mutual relations, and moreover, a considerable variability in the measured quantities was expected between individuals, 'robust' statistical methods were chosen. By 'robust' we mean that the number of assumptions needed to be met to obtain valid results is kept to a minimum, and therefore the results remain reliable under a wide variety of conditions. For example, unlike more commonly used techniques of analysis, such as linear regression, analyses are still valid when variables are non-normally distributed, or when they are related in a non-linear manner, or when there is strong multicollinearity. In addition, due care was taken to adjust for multiple testing. In particular, all the findings mentioned below have gone through the Benjamini Hochberg method [32] that was applied separately to each association study at a False Discovery Rate (FDR) of 15%. This means that, roughly speaking, of all the findings here reported at most 15% could be spurious.

We first focused on our secondary objective, which was to study how immune cell subpopulations differ between the sexes and between CMV seropositive and seronegative individuals, while taking age into account. Therefore, we carried out an association study to detect associations between sex and each of the subsets of immune cells, and between CMV serostatus and each of the subsets of immune cells. Differences according to sex and CMV serostatus with respect to each leukocyte phenotype were tested with the permutation version of the Wilcoxon-Mann-Whitney test [33], blocked by age in four age groups to reduce possible confounding effects of age. For the comparison between sexes, the age groups were further subdivided according to CMV serostatus. Since we used a permutation approach, P values were calculated using 10^8 simulations to estimate the distribution under the null hypothesis. In addition, a Spearman's rho was calculated separately, and defined as the weighted average of Spearman's rho within each block. This was done as Spearmans' rho, not used in calculating p values (which was done by simulations), is useful for estimating the strength of an association. This type of analysis has the advantage over more commonly used tests that no pre-defined assumptions need to be made concerning, for example, the distribution of the data. In addition, ties in ranking, that can occur when ranking variables, such as a frailty index score, do not pose a concern when calculating p values using a permutation approach [34].
Secondly, we focused on our main objective, which was to study how frailty relates to numbers of immune cell subsets and how this relationship differs between the sexes, while taking age and CMV serostatus into account. For this purpose, two complementary analyses were carried out: (A) separate association studies for men and women to detect associations between frailty and each of the cell subsets, and (B) separate analyses for men and women in which a participant's frailty index was predicted based on age, CMV serostatus and date of flow cytometry measurement, in combination with the participant's immune cell numbers. For (A), associations between frailty and each subset of immune cells were assessed with the permutation version of the Spearman correlation test [33] blocked by eight strata based on four age categories and CMV serostatus. P-values were estimated and Spearmans' rho calculated in a similar manner as with the association studies described above. For (B), a random forest predictor was used [35] chiefly with the purpose of obtaining

a ranking of variable importance and to complement the association studies by considering several variables simultaneously. Roughly speaking, in a random forest the importance of a variable is measured in terms of the percentage increase in mean-square error (MSE) that results from randomly shuffling the values of that variable in the data set, so that greater importance is synonymous with greater increase in MSE; see [35] for details. Furthermore, the prediction analysis was assessed in terms of the explained variance. Then, in order to provide a qualitative idea of the relationships between the most important predictor variables and the frailty index, partial dependence plots were created with the pdp R package [36]. This random forest algorithm is known for its accuracy in complex analyses[37, 38, 39]. Therefore, it has been used in a wide variety of research disciplines when many explanatory variables are involved [40, 41, 42, 39]. The algorithm is robust and reliable since, unlike more traditional methods like linear regression models, it can handle multiple explanatory variables that do not need to be normally distributed [37] such as the cell subpopulations in this study. Other advantages of the random forest algorithm when compared to more traditional methods are that it even performs well if strong multicollinearity exists between explanatory variables and that the model does not tend to overfit when using multiple explanatory variables [37, 35].

3 Results

3.1 Study population

The study population consisted of 145 men and 144 women. Average time between blood sample collection and frailty index measurement was 1.6 years (95%CI: 1.4-1.8 years, min: 2 days, max: 4.25 years). Since n=5 men and n=7 women did not participate in the latest DCS assessment round and therefore had a missing frailty index score, these participants were removed from the analyses with frailty.

3.2 Associations between leukocyte subpopulations and sex

An overview of immune cell phenotypes considered in this study is given in Figure 1, all of them measured in peripheral blood. Most of the analyzed subpopulations belong to the T cell lineage. Neutrophils accounted for the highest median numbers in peripheral blood of both men and women, followed by T cells (Figure 2). Women had lower numbers of $CD16^{-}$ (classical) monocytes but higher numbers of B cells and T cells than men. The higher numbers of T cells in women were mainly due to higher $CD4^{+}$ T cell numbers. Further subsetting revealed higher concentrations of almost all CD4 T cell subpopulations (naïve CD4 T cells, central memory CD4 T cells, CD4 Tem and TemRA cells, (naïve) regulatory T cells, and Follicular helper T cells) in peripheral blood of women. This pattern was also seen in early differentiated CD4 subsets but, in contrast, not in late differentiated CD4 Tem cells which showed lower numbers in women (Figure 3). Furthermore, we also observed lower numbers of $CD56^{+}$ T cells in women. While total numbers of $CD8^{+}$ T cells were not found to be different between the sexes, CD8 naïve T cell numbers were found to be higher in women (Figure 2). More detailed phenotyping of CD8 T cell subpopulations revealed higher numbers of early differentiated CD8 Tem and TemRA cells in women than in men, but lower numbers of late differentiated CD8 Tem and TemRA cells (Figure 3).

3.3 Associations between leukocyte subpopulations and CMV serostatus

CMV seropositivity was associated with higher numbers of T cells and $CD56^+$ T cells in men and in women (Table S2). Numbers of $CD8^+$ T cells were observed to be higher in CMV seropositive (CMV^+) men and CMV^+ women, which was mainly due to higher numbers of late stage $CD8^+$ Tem and TemRA cells. Numbers of $CD4^+$ T cells were observed to be higher in CMV^+ men but not in CMV^+ women. However, late stage $CD4^+$ Tem and TemRA cells were higher in CMV^+ men and CMV^+ women. Also, the CD4/CD8 ratio was lower in CMV seropositive individuals of both sexes, indicating that the higher T cell numbers in CMV^+ participants was more due to the higher numbers of $CD8^+$ T cells rather than $CD4^+$ T cells. In men, CMV seropositivity was also associated with higher numbers of follicular helper T cells and with lower numbers of monocytes due to lower numbers of $CD16^-$ (classical) monocytes. These classical monocytes also showed higher CD38 expression.

3.4 Relationship of frailty with leukocyte subpopulations

In the association study, an association between frailty and higher neutrophil numbers was found in men and in women (tables S3 and S4), while other associations were observed only in women. Frailer women, but not men, showed higher monocyte numbers, in particular classical monocyte numbers. In addition, frailer women also showed lower $CD56^+$ T cell numbers and lower $CD4^+$ TemRA cell numbers. Interestingly, frailty was not found to be associated with any other T cell subpopulation and thus, not with most memory T cell subpopulations.

In the prediction analysis with the random forest algorithm, predictive power turned out to be weak in both of the sexes with the percentage of explained variance being 9.2% in men and 12.2% in women. Variable importance of the subpopulations showed that neutrophils were the only subpopulations among the most important variables in the model to predict frailty in men and in women (Figure 4, with 'most important' variables, defined as >50% increase in MSE, shown in bold). Other relationships between frailty and the immune cell profile were sex-specific. The subpopulations with highest increase in MSE were neutrophils in women and $CD16^+$ monocytes in men. In women, other monocyte subpopulations were found among the most important subpopulations related to frailty, namely total monocytes and $CD16^-$ monocytes. Furthermore, among the most important subpopulations related to frailty in men were also naïve B cells and plasmablasts, and in women $CD56^+$ T cells and total T cells. Age was also an important variable to predict frailty in both sexes. Interestingly, CMV serostatus was not important in the prediction model, with an increase in MSE of less than 0.5% in either of the sexes.

An overall impression of the strength and quality of the role of the more important variables in predicting frailty is provided by the partial dependence plots shown in Figure 5. These show the 'effect' of a cell type on frailty when the other variables in the random forest predictor are held constant at average values. The clearer and stronger relationships with frailty were seen in the myeloid cell numbers. In accordance with the association study, an increase in neutrophil numbers was seen with a higher frailty index in both men and women. An increase in numbers of classical monocytes with frailty was only seen in women, and an increase in non-classical monocytes with frailty was only seen in men. Moreover, frailer men but not

women demonstrated lower naïve B cell numbers and frailer women showed lower CD56+ T cells. The direction of the relationship between frailty and plasmablast numbers in men was less clear, with the partial dependence plot showing a possible non-monotonous 'U-shaped' relationship.

3.5 Associations between frailty and proportions of leukocyte subpopulations

We additionally investigated whether frailty was associated with relative values (percentages) of immune cell subpopulations within the major parent populations (that is, within T cells, B cells, monocytes, and NK cells). In this analysis, no associations with frailty were detected (Tables S5 and S6).

4 Discussion

This study shows that frailty in a 60-87-year-old population was associated with higher absolute numbers of neutrophils in men and women, and also with several sex-specific changes in the immune cellular profile. Frailer women demonstrated higher numbers of total monocytes and $CD16^-$ monocytes and lower numbers of $CD56^+$ T cells in both an association study and in a prediction analysis. We also observed a negative association between frailty and late differentiated $CD4^+$ TemRA cells, but this result was not confirmed in the prediction analysis. Overall, the predictive value of the random forest model was low. Yet, the random forest model revealed additional sex-specific relationships with frailty, showing that higher frailty index scores were related to higher $CD16^+$ monocyte and lower naïve B cell numbers in men. Notably, we detected no positive associations between frailty and memory T cell subpopulations.

To the best of our knowledge, this is the first study in which frailty was investigated for associations with different monocyte subpopulations. The most abundant monocytes are the classical $CD16^-$ monocytes. These have clear phagocytic capacity, can evolve into macrophages [43] and therefore are involved in defense against pathogens. Classical monocytes can also evolve into a rarer subpopulation, the non-classical monocytes [44]. Our $CD16^+$ monocyte subpopulation consists of both non-classical and intermediate monocytes, of which non-classical monocytes are thought to be the most matured subset [44]. Evidence is conflicting about whether non-classical monocytes have a pro-inflammatory [43, 45] or a more regulatory role [46]. However, they seem to be involved in vascular homeostasis and atherosclerosis by patrolling vascular endothelia [47], and their numbers are elevated in numerous other diseases and conditions like sepsis [43], arthritis [48], and atherosclerosis [47]. This indicates that they play an important role in mediating health and disease. In addition, non-classical monocytes seem to be involved in vascular homeostasis by patrolling vascular endothelia and controlling plaque formation in atherosclerosis [47]. The fact that conditions like atherosclerosis are more common in men, possibly due to a 'protective' effect of estrogen in women [49], might help to explain why we observed higher numbers of non-classical monocytes in frailer men. We also observed lower numbers of T cells expressing the NK cell marker CD56 in frailer women, but not men. The relationship of frailty with $CD56^+$ T cells has not been described earlier. However, in one study NK cell markers (CD16 and CD56) were shown to be more highly expressed by T cells from people with better cognitive and physical

functioning [50], a finding that corresponds to the negative relationship found in our study between $CD56^+$ T cell numbers and frailty in women. Furthermore, in the prediction analysis we observed other relationships between frailty and the immune cellular profile, such as lower naïve ($CD19^+CD38^{DIM}CD27^-$) B cell numbers in frailer men, and a non-monotonous relationship between frailty and plasmablasts in men, but not women. Sex-specific associations between frailty and B cell subpopulations were described in a recently published paper, in which the authors observed that men with a higher frailty index score had higher double negative ($CD27^-$ and IgD^-) memory B cell percentages [51]. Although we did not use IgD to distinguish the $CD27^-IgD^-$ memory B cell subset from the naïve $CD27^-$ B cell population, it could be that a higher proportion of double negative memory B cells, as observed by Nevalainen et al., is explained by lower absolute numbers of $CD27^+IgD^+$ naïve B cells, which would be in line with our findings, a notion to be further investigated. Thus, frailer men showed higher non-classical monocyte numbers and possibly lower naïve B cell numbers, while frailer women showed higher classical monocytes and lower $CD56^+$ T cells. These findings could be of interest in relation to the sex-frailty paradox, namely that women on average are frailer than men but tend to live longer [52]. Further insight may eventually lead to a better understanding of the mechanisms underlying this paradox.

The associations found between higher myeloid cell numbers and frailty are in line with previous reports [5, 53, 3]. In one study it was shown that elevated neutrophil levels in humans are related to negative health outcomes [54], which corresponds to our results, since frail people are more prone to adverse outcomes like early mortality. In a murine study, it was argued that both higher myeloid cell abundancies and lower vaccine responses in older mice could be the result of intrinsic differences in hematopoietic stem cells [55]. Other studies suggest that the myeloid bias of hematopoietic stem cell differentiation seen in old age is due to a chronic low-grade inflammation [56]. This process is possibly regulated by plasma cells in bone marrow that can produce pro-inflammatory cytokines that are shown to be related to myelopoiesis [57]. Such a chronic low-grade inflammation is often seen in frail people [58]. However, many other factors may impact cellular numbers in peripheral blood, like variations in extravascular homing, or differential apoptosis [59].

Interestingly, in this study no positive associations were found between frailty and naïve- and memory T cell subpopulations in either of the sexes. This is of interest because it is known that the balance between naïve and memory T cells in peripheral blood changes with advancing age, mainly due to lower numbers of naïve ($CD8^+$) T cells [60, 61, 11], which was also observed in our study (data not shown). However, instead of a positive association, we observed a negative association between late differentiated $CD4^+$ TemRA cells and frailty in women. But since it was the weakest observed association in women and the association was not confirmed in the random forest analysis, the importance of this result requires confirmation in future studies. Previous studies, analyzing fewer immune cell subpopulations, did not detect associations between frailty and naïve- and memory T cell subpopulations [5, 7]. However, contrasting results were found in the Singapore Longitudinal Aging Study population, in which associations were found between frailty and memory

T cell subpopulations, as well as with CD4/CD8 ratio [6, 62]. But the results of these studies were based on the analysis of a relatively small frail population (32 participants) without adjustments for cytomegalovirus (CMV) infection, which is well known to generate clonal expansion of memory T cells and thus may have confounded this study. In our study we confirmed previously found associations of CMV seropositivity with the late-stage memory T cell population [25, 11]. We also observed that CMV seropositive men had lower classical monocyte numbers. In addition, their classical monocytes seemed to have higher CD38 expression. It is thought that CD38 can be upregulated in monocytes and macrophages in inflammatory conditions [21]. Further research is needed to confirm this association and to answer the question why it was only found in men. Furthermore, we observed that CMV serostatus was not important in our frailty prediction model. Conflicting evidence has been reported on whether there is a positive relationship between CMV serostatus and frailty, with some studies showing an association [63, 64] while others do not [5, 65]. These conflicting results might be explained by differences in the frailty instruments used (Frailty phenotype or frailty index) or differences in study population, with some studies being restricted to only one of the sexes [63] or to extreme elderly [5, 65].

The present study also shows that several immune cell subpopulations differ in abundancy between the sexes, with higher numbers of most T cell subpopulations, especially CD4 T helper cells, and higher numbers of B cells in women. These findings are in line with previous studies [25]. 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 [66, 67]. In addition, immune function differs between the sexes, with women showing better responses to antigens and a stronger tendency to develop autoimmune diseases [52, 66].

One of the strengths of this study is the use of fresh whole blood samples to measure an extensive set of immune cell subpopulations. This made it possible to enumerate cell populations like neutrophils that are not possible to quantify when using cryopreserved PBMCs. In addition, we were able to relate absolute numbers of 37 immune subsets to frailty. This is important as these are needed to correctly interpret relative values (e.g. percentages). As an example, a change in relative values can be the result of a change in either the numerator or the denominator. This issue can be solved only with information on absolute numbers. Our study also showed that, when we performed an analysis with subpopulations of B cells, T cells, NK cells, and monocytes expressed as percentages, this analysis did not give additional insight in how frailty relates to the immune cellular profile. Another strength of our study is that we used a robust statistical analysis framework with two different analytical approaches that complement each other to find associations with frailty while correcting for multiple testing. In our statistical analysis framework, we also adjusted for age and CMV serostatus in the association study between frailty and the immune cellular profile by using four age groups and CMV serostatus as blocking factors. Moreover, another noteworthy asset of our study is that it concerned a sample from the general community-dwelling population, thus allowing a window

on aging of the immune system in a non-clinical setting. On the other hand, due to the nature of using fresh whole blood samples and our aim being to quantify a large number of different immune cell subpopulations, we were restricted in our antibody panel and therefore not all the subsets could be further classified with additional phenotypic markers. For example, to define regulatory T cells we used CD45RA and CD25 as was done previously [23, 24, 25], but not FoxP3 or CD127. In addition, it appeared, as already expected, that the variability in numbers of immune cell subsets is very large in older adults. This may have contributed to the low prediction accuracy observed in the prediction analysis. Furthermore, there is no consensus yet on which instrument is the best to investigate frailty, and different instruments might yield different results. Since we were interested in a measure that approximates general health, we used a Rockwood frailty index [16] based on 36 deficits rather than a frailty instrument with a less broad definition like the Fried frailty phenotype [68].

5 Conclusions

In this study we found associations between frailty and higher neutrophil numbers in both men and women, but not between frailty and higher memory T cell numbers. In-depth immune profiling also revealed sex-specific associations with frailty, with frailer women but not men showing higher numbers of classical monocytes and lower numbers of $CD56^+$ T cells than their healthier peers.. These sex-specific associations, to the best of our knowledge, have not been reported earlier. An expected positive association between frailty and memory T cells was not observed. We hope that our study will prompt further investigation into the different immune mechanisms associated with the development of frailty in men and women.

Abbreviations

CMV: Cytomegalovirus; DCS: Doetinchem Cohort Study; FDR: False Discovery Rate; Tem: effector memory T cells; TemRA: effector memory T cells re-expressing CD45RA

Acknowledgements

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, and Sara van den Berg, Marjan Bogaard, and Irina Tcherniaeva for their help with the cytomegalovirus assay.

Authors' 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, LdR, and LS developed the flow cytometry laboratory protocols and performed the experimental work in the laboratory. JF provided the concept of the data analysis framework, and LS, JF, and PE implemented it in the manuscript. LS, PE, JF, AB, MB, and MV interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

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.

Availability of data and materials

The datasets used are available from the corresponding author upon reasonable request and with permission of the scientific committee of the Doetinchem Cohort Study.

Ethical 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

Competing Interest
The authors declare that they have no competing interests

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Figures

Tables

Additional Files

Additional file 1 — Supplementary Figures S1-S3 and Tables S1-S4

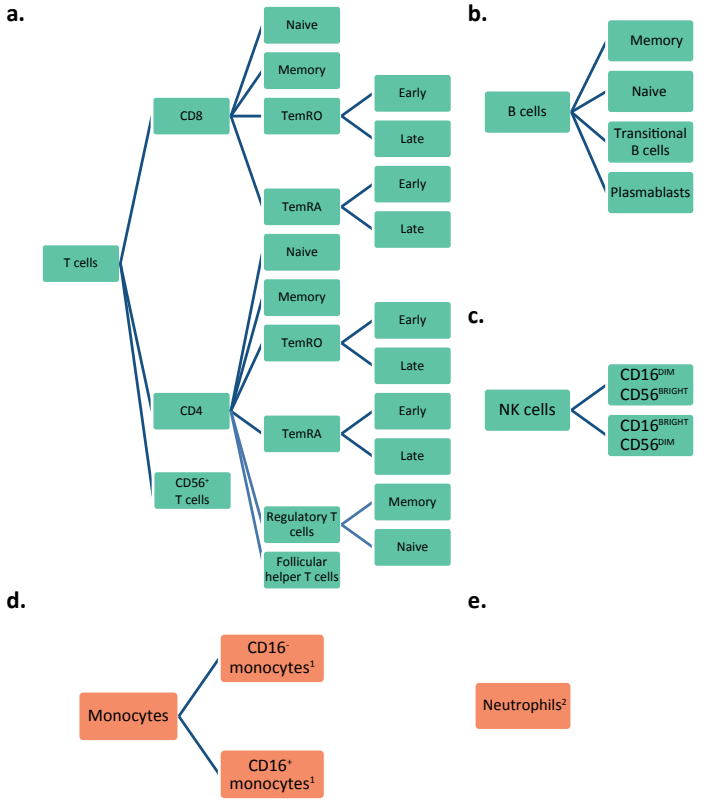

Figure 1 Subpopulations of (a) T cells, (b) B cells, (c) NK cells, (d) Monocytes, and (e) Neutrophils, of which absolute cell numbers were determined. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. See Table S1 for definition of all phenotypes.
[1]Additionally analyzed on expression of CD38 and HLA-DR.
[2]Additionally analyzed on expression of CD16.

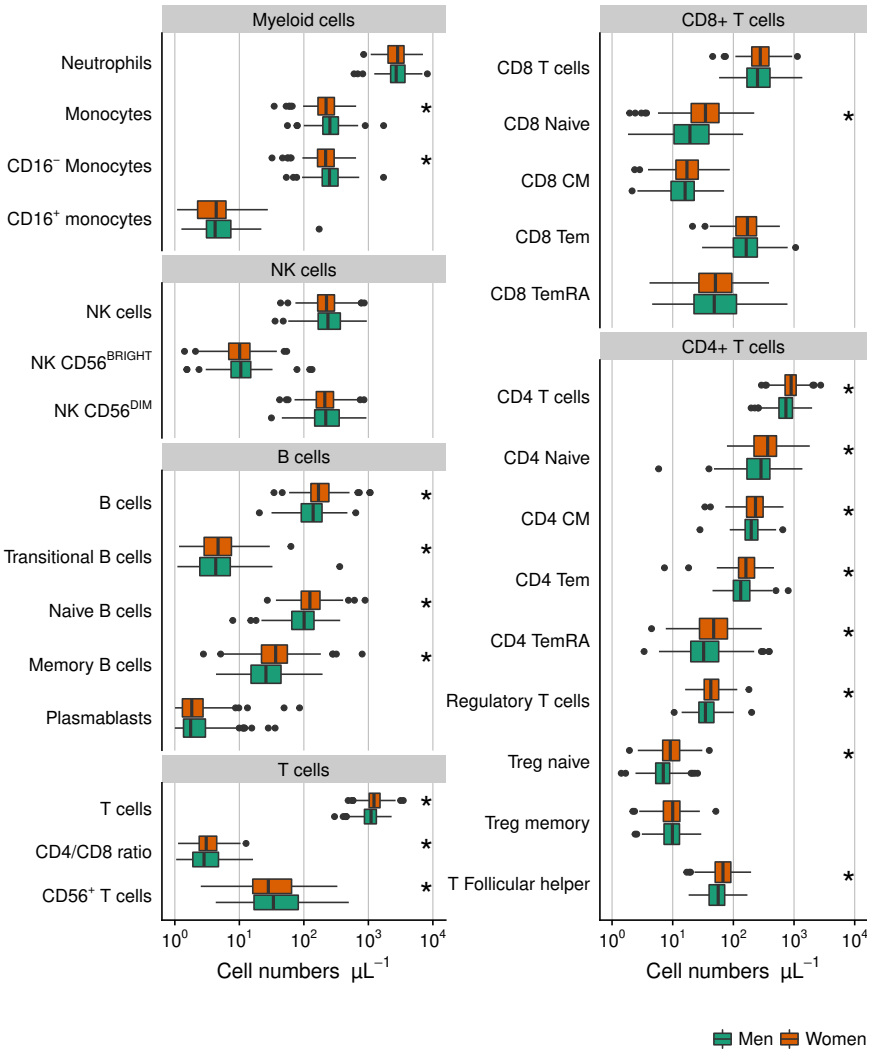

Figure 2 The numbers of cells per leukocyte subpopulations in men (n=145) and women (n=144) above 60 years of age. The boxplots show median values with interquartile range. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. *: Outcomes of tests of the association between leukocyte numbers and sex (adjusted for age and CMV serostatus) which passed the Benjamini-Hochberg method at a false discovery rate of 15%.

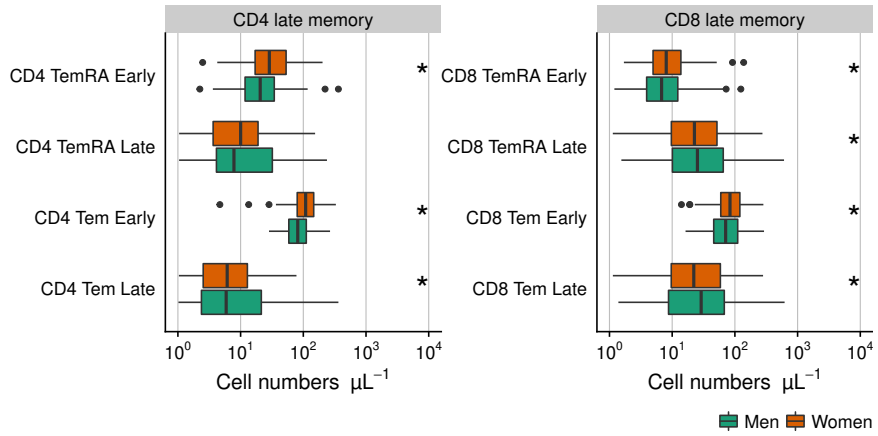

Figure 3 The numbers of CD4 and CD8 Tem and TemRA cells in men (n=145) and women (n=144) above 60 years of age. The boxplots show median values with interquartile range. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. *: Outcomes of tests of the association between leukocyte numbers and sex (adjusted for age and CMV serostatus) which passed the Benjamini-Hochberg method at a false discovery rate of 15%.

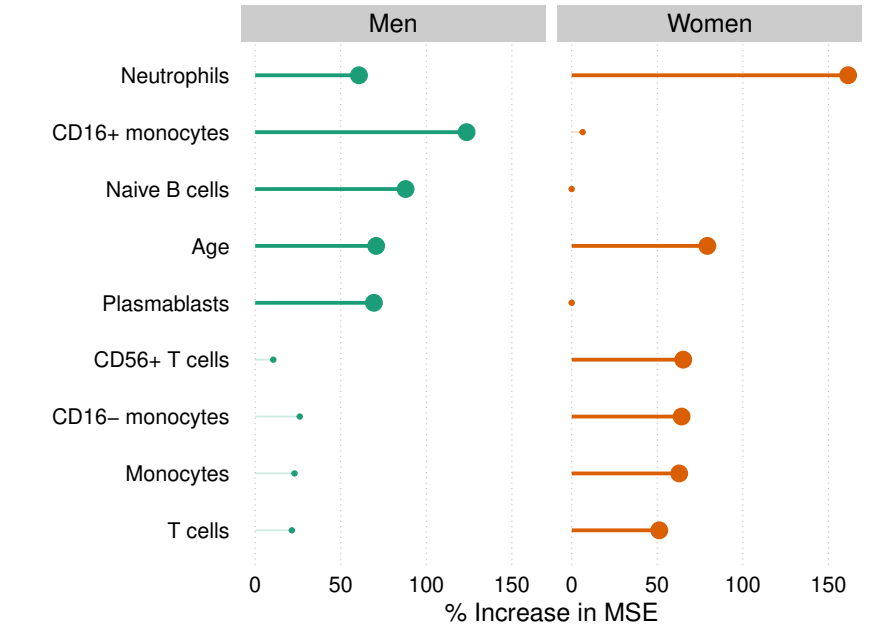

Figure 4 Variable importance plot showing the leukocyte subpopulations and other variables that are most helpful in predicting frailty in men (n=140) and women (n=137). Participants with missing frailty index score data (n=12) were excluded from analysis. The bold lines and dots represent the most important variables that show an increase in mean squared error (MSE) of >50%. See text for the meaning of variable importance.

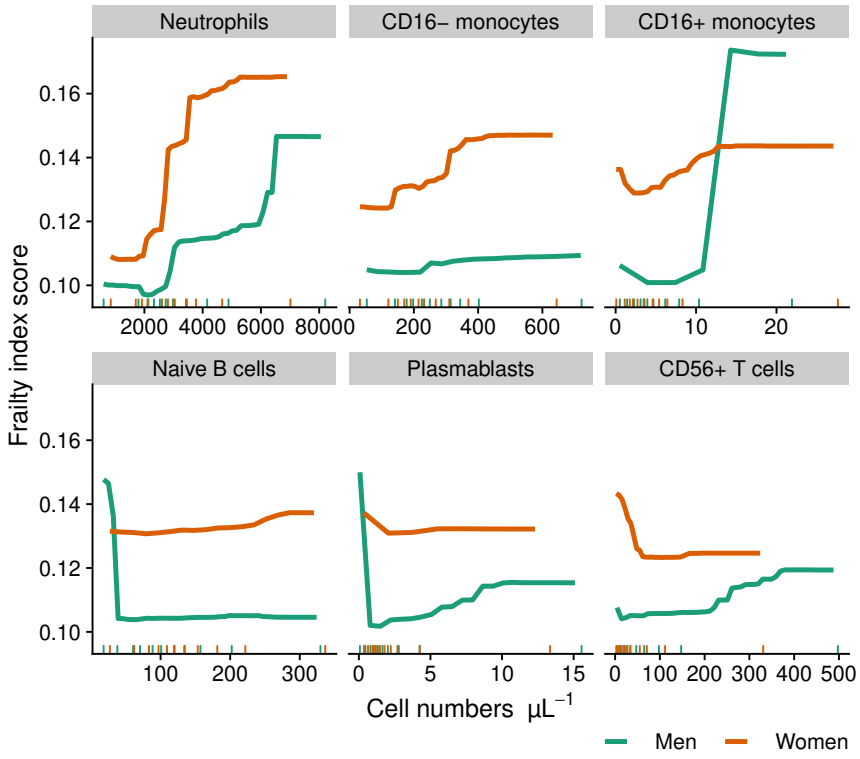

Figure 5 Partial dependence plots illustrating the role of the most important leukocyte phenotypes in predicting frailty in a random forest predictor for men (n=140) and women (n=137). Participants with missing frailty index score data (n=12) were excluded from analysis. The short vertical segments on the horizontal axis represent the deciles of the cell numbers in the data. Range of the figures is restricted to the part containing most of the data.

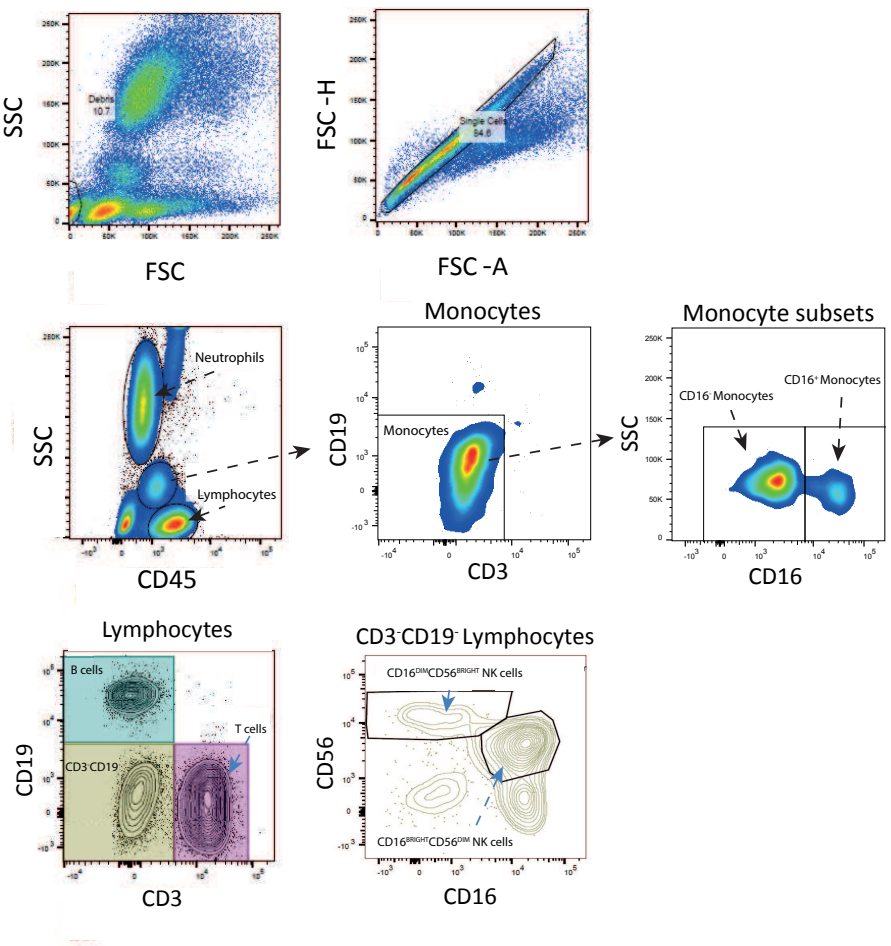

Figure S1 General gating strategy showing gating of single cells by FSC-A versus FSC-H, leukocyte subsets by SSC and CD45, monocyte subpopulations by CD16, T and B-cells by CD3 and CD19, and NK cells by CD16 and CD56.

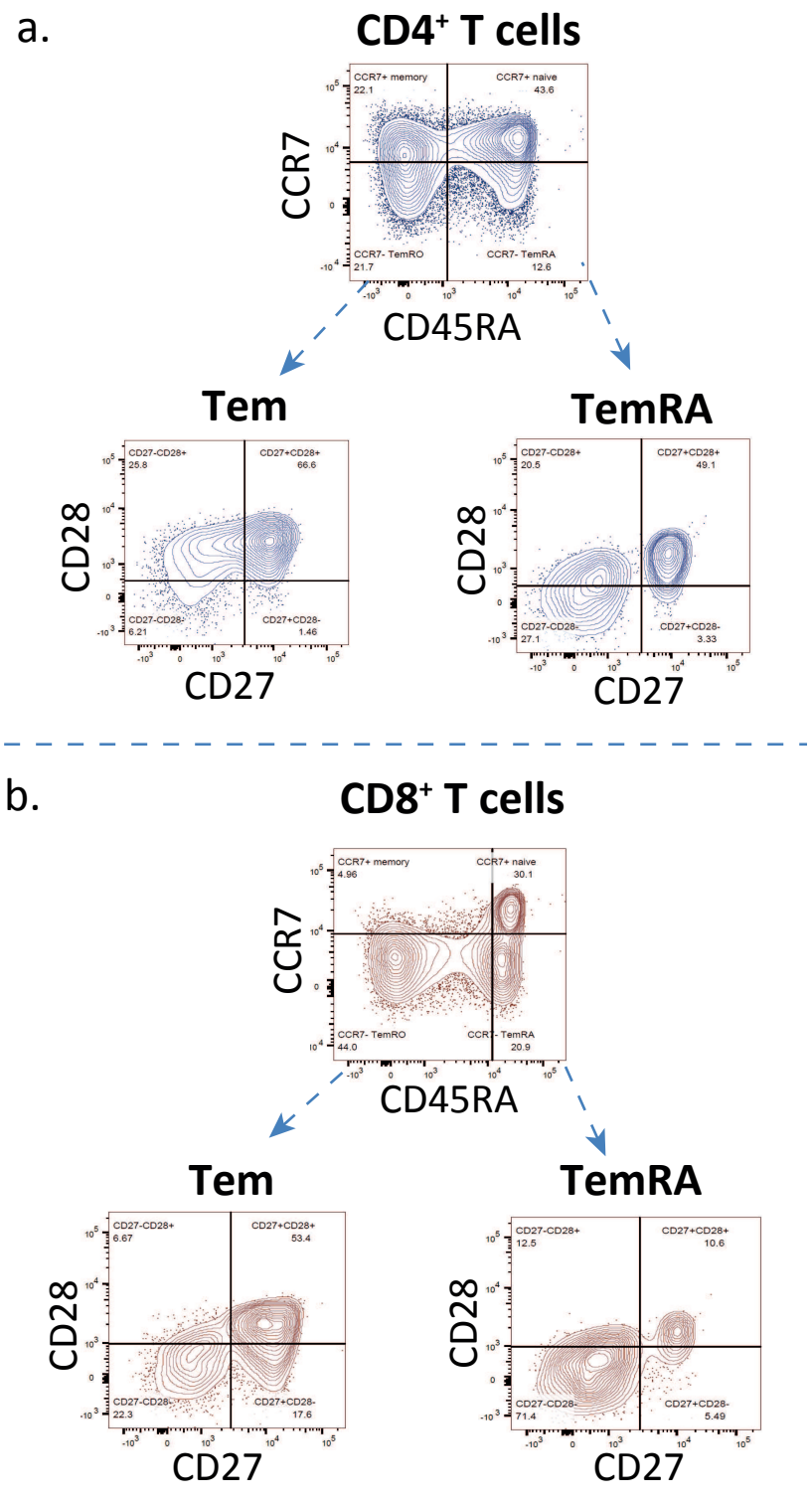

Figure S2 Gating strategy for (a) memory CD4 T cells and (b) memory CD8 T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: Terminally differentiated (CD4 or CD8 positive) T cells re-expressing CD45RA.

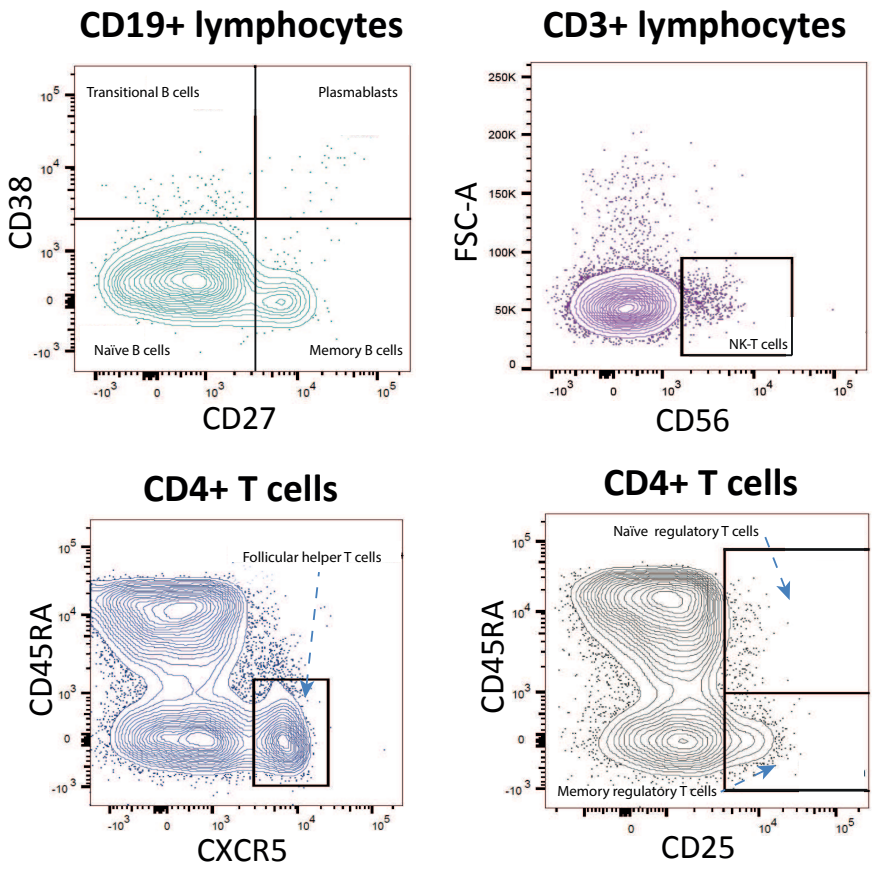

Figure S3 Gating strategy for (a) B cell subsets and (b) follicular helper T cells and regulatory T cells.

Table S1 Definition of cell phenotypes by expression of cell surface markers

Cell phenotype	Definition
T cells	
T cells	$CD45^{+}CD3^{+}$
T Follicular helper	$CD3^{+}CD4^{+}CD45RA^{-}CXCR5^{+}$
$CD56^{+}$ T cells	$CD45^{+}CD3^{+}CD56^{+}$
CD4/CD8 ratio	CD4 T cells/CD8 T cells
CD4 T cells	$CD45^{+}CD3^{+}CD4^{+}$
CD4 Naive	$CD3^{+}CD4^{+}CD45RA^{+}CCR7^{+}$
CD4 CM	$CD3^{+}CD4^{+}CD45RA^{-}CCR7^{+}$
CD4 TemRA	$CD3^{+}CD4^{+}CD45RA^{+}CCR7^{-}$
CD4 TemRA Early	$CD3^{+}CD4^{+}CD45RA^{+}CCR7^{-}CD27^{+}CD28^{+}$
CD4 TemRA Late	$CD3^{+}CD4^{+}CD45RA^{+}CCR7^{-}CD27^{-}CD28^{-}$
CD4 Tem	$CD3^{+}CD4^{+}CD45RA^{-}CCR7^{-}$
CD4 Tem Early	$CD3^{+}CD4^{+}CD45RA^{-}CCR7^{-}CD27^{+}CD28^{+}$
CD4 Tem Late	$CD3^{+}CD4^{+}CD45RA^{-}CCR7^{-}CD27^{-}CD28^{-}$
Regulatory T cells	$CD3^{+}CD4^{+}CD25^{BRIGHT}$
Treg naive	$CD3^{+}CD4^{+}CD25^{BRIGHT}CD45RA^{+}$
Treg memory	$CD3^{+}CD4^{+}CD25^{BRIGHT}CD45RA^{-}$
CD8 T cells	$CD45^{+}CD3^{+}CD8^{+}$
CD8 Naive	$CD3^{+}CD8^{+}CD45RA^{+}CCR7^{+}$
CD8 CM	$CD3^{+}CD8^{+}CD45RA^{-}CCR7^{+}$
CD8 TemRA	$CD3^{+}CD8^{+}CD45RA^{+}CCR7^{-}$
CD8 TemRA Early	$CD3^{+}CD8^{+}CD45RA^{+}CCR7^{-}CD27^{+}CD28^{+}$
CD8 TemRA Late	$CD3^{+}CD8^{+}CD45RA^{+}CCR7^{-}CD27^{-}CD28^{-}$
CD8 Tem	$CD3^{+}CD8^{+}CD45RA^{-}CCR7^{-}$
CD8 Tem Early	$CD3^{+}CD8^{+}CD45RA^{-}CCR7^{-}CD27^{+}CD28^{+}$
CD8 Tem Late	$CD3^{+}CD8^{+}CD45RA^{-}CCR7^{-}CD27^{-}CD28^{-}$
Neutrophils	
Neutrophils	$SSC^{BRIGHT}CD45^{DIM}$
Neutrophils, CD16 expr.	$SSC^{BRIGHT}CD45^{DIM};CD16^{expression}$
Monocytes	
Monocytes	$SSC^{DIM}CD45^{+}CD3^{-}CD19^{-}$
$CD16^{-}$ monocytes	$SSC^{DIM}CD45^{+}CD16^{-}$
$CD16^{-}$ mon. CD38 expr.	$SSC^{DIM}CD45^{+}CD16^{-};CD38^{expression}$
$CD16^{-}$ mon. HLADR expr.	$SSC^{DIM}CD45^{+}CD16^{-};HLADR^{expression}$
$CD16^{+}$ monocytes	$SSC^{DIM}CD45^{+}CD16^{+}$
$CD16^{+}$ mon. CD38 expr.	$SSC^{DIM}CD45^{+}CD16^{+};CD38^{expression}$
$CD16^{+}$ mon. HLADR expr.	$SSC^{DIM}CD45^{+}CD16^{+};HLADR^{expression}$
B cells	
B cells	$CD45^{+}CD19^{+}$
Plasmablasts	$CD45^{+}CD19^{+}CD38^{BRIGHT}CD27^{BRIGHT}$
Transitional B cells	$CD45^{+}CD19^{+}CD38^{BRIGHT}CD27^{-}$
Naive B cells	$CD45^{+}CD19^{+}CD38^{DIM}CD27^{-}$
Memory B cells	$CD45^{+}CD19^{+}CD38^{DIM}CD27^{+}$
NK cells	
NK cells	Either NK $CD56^{BRIGHT}$ or NK $CD56^{DIM}$
NK $CD56^{BRIGHT}$	$CD45^{+}CD3^{-}CD19^{-}CD16^{DIM}CD56^{BRIGHT}$
NK $CD56^{DIM}$	$CD45^{+}CD3^{-}CD19^{-}CD16^{BRIGHT}CD56^{DIM}$

Table S2 Leukocyte numbers per sex and CMV serostatus

	Men			Women		
	CMV- (n=69)	CMV+ (n=76)	P value[1]	CMV- (n=53)	CMV+ (n=91)	P value[1]
T cells						
T cells	1002.6 (428.9)	1202.5 (463.4)	<0.001*	1144.7 (436.2)	1272.4 (474.9)	0.026*
T Follicular helper	49.5 (28.8)	61.8 (34.1)	0.04*	67.5 (37.6)	66.7 (40.9)	0.374
$CD56^+$ T cells	21.8 (27.8)	60.9 (108.5)	<0.001*	16 (16.7)	48.5 (61.9)	<0.001*
CD4/CD8 ratio	3.6 (2.7)	2.5 (1.8)	0.002*	4.2 (3.2)	2.8 (1.7)	<0.001*
CD4 T cells	669.7 (370.9)	766.5 (391.6)	0.041*	897.3 (371.9)	892.2 (363.3)	0.847
CD4 Naive	316.3 (283.4)	243.1 (215.2)	0.292	343.7 (262)	374.6 (295.9)	0.924
CD4 CM	175.6 (90.3)	217.1 (105)	0.01*	234.1 (118)	230.9 (149.4)	0.179
CD4 TemRA	27.8 (28.8)	46.7 (45.7)	<0.001*	33.9 (50.7)	52.6 (52.4)	0.003*
CD4 TemRA Early	22.4 (26.5)	19.7 (21.2)	0.666	29.7 (44.1)	28.2 (32.2)	0.839
CD4 TemRA Late	0.1 (0.2)	5.4 (24.2)	<0.001*	0.1 (0.2)	5.9 (13.9)	<0.001*
CD4 Tem	110.6 (70.1)	153.8 (108.3)	<0.001*	155.8 (91.6)	179.9 (112.8)	0.177
CD4 Tem Early	77.7 (51.5)	86.9 (54.3)	0.166	114.7 (59.8)	106 (73.2)	0.362
CD4 Tem Late	1.1 (1.5)	15 (25.5)	<0.001*	1.1 (1.6)	8.9 (14.2)	<0.001*
Regulatory T cells	34 (18.8)	34.7 (23.8)	0.556	42.6 (22.8)	42.1 (26.5)	0.377
Treg naive	6.7 (4.1)	7.2 (3.2)	0.607	8.3 (6.4)	9.4 (5.7)	0.479
Treg memory	9.3 (5.8)	10.2 (5.2)	0.343	10.5 (5.6)	9.1 (6.4)	0.049
CD8 T cells	188.9 (198.7)	337.2 (246.1)	<0.001*	203.5 (124.1)	333.2 (159.1)	<0.001*
CD8 Naive	20 (31.5)	18 (26.4)	0.532	35.4 (41.9)	34.1 (33.8)	0.948
CD8 CM	13.7 (11.7)	17.3 (15.6)	0.01*	17.3 (14)	16.8 (15.3)	0.76
CD8 TemRA	32.3 (36.9)	83.6 (109.9)	<0.001*	27.8 (26.9)	75 (74)	<0.001*
CD8 TemRA Early	6.3 (7.8)	7.4 (8.4)	0.73	6.7 (7.5)	9.1 (9.3)	0.129
CD8 TemRA Late	10.5 (16.1)	54.1 (66.2)	<0.001*	8.6 (13)	40.4 (49.1)	<0.001*
CD8 Tem	120.1 (116.9)	227.9 (135.7)	<0.001*	117.4 (62.9)	212.4 (111.9)	<0.001*
CD8 Tem Early	64.3 (63.6)	73 (68.1)	0.409	74.7 (53.3)	88.6 (59.3)	0.538
CD8 Tem Late	11.4 (14.5)	59 (72.8)	<0.001*	6.6 (7.8)	48.9 (55.5)	<0.001*
Neutrophils						
Neutrophils	2811.5 (2204.9)	2646.3 (1309.4)	0.197	2707.9 (1478.6)	2851.3 (1573.1)	0.733
Neutrophils, CD16 expr.[2]	3671 (5564)	3971 (5900.2)	0.393	3950 (5273)	4343 (5299)	0.86
Monocytes						
Monocytes	276.5 (136.6)	246.5 (126.3)	0.057*	233.4 (144.2)	218.4 (125.2)	0.365
$CD16^-$ monocytes	273.2 (134.9)	246 (122.1)	0.064*	229.3 (143)	211.8 (125.8)	0.351
$CD16^-$ mon. CD38 expr.	1200.5 (338.2)	1273 (341.5)	0.039*	1168 (193)	1216 (285)	0.401
$CD16^-$ mon. HLADR expr.	9106 (6090.2)	8736 (4603)	0.78	8834 (3806)	8171 (6208)	0.907
$CD16^+$ monocytes	4 (4.7)	4 (3.9)	0.397	4 (3.8)	3.4 (4.2)	0.628
$CD16^+$ mon. CD38 expr.	445.5 (192.5)	499 (199.5)	0.157	412 (139)	444 (218)	0.138
$CD16^+$ mon. HLADR expr.	56851 (27956)	52916 (41323.5)	0.587	42036 (41637)	39669 (39328)	0.935
B cells						
B cells	124 (86.8)	150.4 (104.2)	0.427	167.7 (115.2)	173.9 (118.8)	0.904
Plasmablasts	1.5 (1.5)	1.4 (1.4)	0.677	1.5 (1.5)	1.4 (1.2)	0.644
Transitional B cells	3.7 (3.7)	3.8 (5.6)	0.643	4.6 (4.8)	4.2 (4.4)	0.806
Naive B cells	92.1 (72.8)	111 (75.6)	0.487	124.8 (88.9)	120.4 (78.1)	0.826
Memory B cells	24 (24.4)	28.4 (30)	0.73	32.8 (33.4)	37.1 (32.2)	0.667
NK cells						
NK cells	274.9 (217)	217.4 (191.2)	0.109	236.1 (117)	212.3 (138.8)	0.39
NK $CD56^{BRIGHT}$	10.7 (7.2)	10.4 (7.7)	0.872	11.6 (8.9)	9.9 (6.6)	0.199
NK $CD56^{DIM}$	251 (219.4)	203.1 (183.8)	0.116	220.8 (109.8)	204.6 (139.5)	0.46

Note:

All values are median (interquartile range) cell numbers per µL, unless otherwise stated

[1] P values of Kruskal-Wallis rank sum test between CMV serostatus per sex, adjusted for age

[2] Values in Median fluoresence intensity

* Selected outcomes when the False Discovery Rate is set to a maximum of 15%

Table S3 Spearman associations between immune cell subpopulations and frailty in men (n=140)

Immune cell subset	Spearman's rho	P value	FDR*
Neutrophils	**0.245**	**0.002**	**0.077**
Transitional B cells	-0.188	0.015	0.324
Monocytes	0.166	0.027	0.389
$CD16^-$ monocytes	0.155	0.036	0.383
CD4 Naive	0.172	0.040	0.341
CD4 TemRA Late	0.129	0.078	0.562
CD4 TemRA	0.166	0.090	0.553
Regulatory T cells	0.122	0.104	0.561
$CD16^+$ monocytes	0.162	0.133	0.634
$CD16^-$ mon. HLADR expr.	-0.145	0.133	0.571
NK $CD56^{DIM}$	-0.079	0.172	0.672
CD4 TemRA Early	0.136	0.191	0.686
CD4 T cells	0.088	0.207	0.683
NK cells	-0.061	0.208	0.638
CD4/CD8 ratio	0.103	0.219	0.628
Memory B cells	-0.072	0.319	0.857
T cells	0.087	0.321	0.812
CD8 Tem Early	-0.067	0.354	0.844
Treg naive	0.077	0.387	0.877
Neutrophils, CD16 expr.	-0.098	0.399	0.858
T Follicular helper	0.070	0.439	0.898
$CD16^+$ mon. CD38 expr.	-0.049	0.440	0.860
Lymphocytes	0.037	0.574	1.073
NK $CD56^{BRIGHT}$	-0.026	0.582	1.042
CD8 Naive	0.053	0.590	1.014
CD8 CM	-0.049	0.650	1.074
CD8 T cells	0.027	0.658	1.048
B cells	-0.056	0.667	1.025
CD8 Tem Late	0.091	0.689	1.022
$CD56^+$ T cells	0.027	0.694	0.995
CD8 Tem	0.015	0.697	0.967
$CD16^-$ mon. CD38 expr.	-0.005	0.705	0.948
$CD16^+$ mon. HLADR expr.	0.071	0.728	0.949
CD4 Tem Late	0.033	0.735	0.930
CD4 CM	-0.051	0.757	0.931
CD4 Tem Early	-0.030	0.789	0.943
CD8 TemRA Late	0.021	0.796	0.925
Plasmablasts	0.040	0.810	0.916
Naive B cells	-0.052	0.839	0.925
CD8 TemRA	0.023	0.851	0.915
CD8 TemRA Early	-0.010	0.931	0.976
Treg memory	0.028	0.967	0.990
CD4 Tem	0.003	0.994	0.994

Note:
The (Spearman) associations are ordered by p value, with the lowest p values shown at the top. Associations that were selected with a FDR lower than 15% are shown in bold.

* False Discovery Rate (estimated)

Table S4 Spearman associations between immune cell subpopulations and frailty in women (n=137)

Immune cell subset	Spearman's rho	P value	FDR*
Neutrophils	**0.399**	**<0.001**	**0.000**
CD16$^-$ monocytes	**0.237**	**0.003**	**0.066**
Monocytes	**0.234**	**0.004**	**0.054**
CD56$^+$ T cells	**-0.198**	**0.01**	**0.109**
CD4 TemRA Late	**-0.131**	**0.015**	**0.128**
CD16$^-$ mon. CD38 expr.	-0.199	0.022	0.157
CD4 Tem Early	0.205	0.032	0.194
CD16$^+$ mon. CD38 expr.	-0.184	0.037	0.200
CD16$^-$ mon. HLADR expr.	-0.116	0.063	0.302
CD4 Tem	0.153	0.073	0.313
NK CD56^{BRIGHT}	-0.150	0.08	0.311
CD8 TemRA	-0.094	0.091	0.325
Transitional B cells	-0.164	0.096	0.318
CD8 TemRA Late	-0.079	0.099	0.303
NK CD56^{DIM}	-0.152	0.133	0.382
NK cells	-0.133	0.149	0.401
CD8 CM	0.145	0.165	0.418
CD4 Tem Late	-0.042	0.235	0.562
CD4/CD8 ratio	0.112	0.25	0.567
CD4 TemRA Early	0.093	0.274	0.589
CD8 T cells	-0.102	0.322	0.658
CD4 CM	0.074	0.34	0.665
CD4 Naive	-0.112	0.344	0.643
T Follicular helper	0.088	0.347	0.621
CD8 Naive	-0.128	0.359	0.618
Treg memory	0.075	0.374	0.618
CD8 TemRA Early	-0.020	0.422	0.672
Memory B cells	-0.093	0.428	0.658
Plasmablasts	-0.071	0.461	0.683
T cells	-0.045	0.49	0.702
CD8 Tem Early	0.121	0.519	0.720
Lymphocytes	-0.023	0.526	0.707
CD16$^+$ monocytes	0.071	0.551	0.718
B cells	-0.061	0.579	0.732
Neutrophils, CD16 expr.	-0.006	0.73	0.897
Treg naive	-0.039	0.779	0.930
CD8 Tem	0.018	0.814	0.946
CD4 TemRA	0.002	0.913	1.033
CD16$^+$ mon. HLADR expr.	0.014	0.946	1.044
CD8 Tem Late	0.010	0.953	1.025
Regulatory T cells	0.012	0.966	1.013
CD4 T cells	0.012	0.967	0.990
Naive B cells	0.002	0.984	0.984

Note:
The (Spearman) associations are ordered by p value, with the lowest p values shown at the top. Associations that were selected with a FDR lower than 15% are shown in bold.

* False Discovery Rate (estimated)

Table S5 Additional analysis in men (n=140) to test associations with frailty when percentages of subpopulations are used instead of absolute numbers

Immune cell subset	Spearman's rho	P value	FDR*
Transitional B cells, (%)	-0.218	0.011	0.373
CD4 Naive, (%)	0.223	0.018	0.302
CD4 CM, (%)	-0.242	0.023	0.260
T cells, (%)	0.159	0.047	0.395
NK cells, (%)	-0.112	0.091	0.621
CD4 TemRA Late, (%)	0.111	0.169	0.958
B cells, (%)	-0.139	0.181	0.880
NK $CD56^{BRIGHT}$, (%)	0.101	0.185	0.787
CD4 Tem, (%)	-0.095	0.186	0.702
CD4 Tem Early, (%)	-0.109	0.204	0.695
CD8 T cells, (%)	-0.110	0.209	0.644
CD4 T cells, (%)	0.116	0.213	0.603
Treg memory, (%)	-0.087	0.215	0.562
NK $CD56^{DIM}$, (%)	-0.080	0.247	0.600
CD4 TemRA, (%)	0.097	0.260	0.589
CD8 Naive, (%)	0.076	0.342	0.727
Regulatory T cells, (%)	0.049	0.425	0.849
CD8 CM, (%)	-0.033	0.464	0.877
CD8 Tem Early, (%)	-0.073	0.469	0.840
CD8 Tem Late, (%)	0.111	0.474	0.806
CD4 TemRA Early, (%)	0.055	0.573	0.927
Plasmablasts, (%)	0.043	0.582	0.900
Memory B cells, (%)	-0.012	0.670	0.990
CD8 TemRA, (%)	0.029	0.676	0.958
Naive B cells, (%)	0.012	0.705	0.958
CD8 TemRA Late, (%)	0.004	0.719	0.940
CD8 TemRA Early, (%)	-0.003	0.769	0.969
T Follicular helper, (%)	-0.032	0.793	0.963
$CD16^{-}$ monocytes, (%)	0.009	0.838	0.983
CD4 Tem Late, (%)	-0.046	0.848	0.961
CD8 Tem, (%)	0.078	0.865	0.948
$CD16^{+}$ monocytes, (%)	-0.006	0.890	0.945
Treg naive, (%)	-0.025	0.934	0.962
$CD56^{+}$ T cells, (%)	-0.032	0.992	0.992

Note:

The (Spearman) associations are ordered by p value, with the lowest p values shown at the top. Associations that were selected with a FDR lower than 15% are shown in bold

* False Discovery Rate (estimated)

Table S6 Additional analysis in women (n=137) to test associations with frailty when percentages of subpopulations are used instead of absolute numbers

Immune cell subset	Spearman's rho	P value	FDR*
$CD56^{+}$ T cells, (%)	-0.178	0.009	0.304
CD4 TemRA Late, (%)	-0.173	0.009	0.156
CD8 Tem, (%)	0.196	0.021	0.236
Transitional B cells, (%)	-0.207	0.022	0.188
CD4 Tem Early, (%)	0.173	0.029	0.195
CD8 Tem Early, (%)	0.176	0.056	0.319
CD8 CM, (%)	0.142	0.062	0.301
CD8 TemRA Late, (%)	-0.088	0.065	0.275
CD4 Tem, (%)	0.155	0.066	0.251
CD8 TemRA, (%)	-0.089	0.075	0.256
Naive B cells, (%)	0.131	0.086	0.264
NK cells, (%)	-0.167	0.093	0.264
CD4 Naive, (%)	-0.121	0.133	0.347
CD4 CM, (%)	0.137	0.138	0.336
CD4 T cells, (%)	0.104	0.205	0.465
Treg memory, (%)	0.073	0.229	0.486
CD4 Tem Late, (%)	-0.046	0.232	0.465
CD4 TemRA Early, (%)	0.100	0.258	0.487
T cells, (%)	0.157	0.293	0.524
CD8 T cells, (%)	-0.095	0.294	0.499
Memory B cells, (%)	-0.082	0.334	0.540
T Follicular helper, (%)	0.099	0.350	0.540
$CD16^{+}$ monocytes, (%)	-0.021	0.458	0.677
Treg naive, (%)	-0.053	0.460	0.651
CD8 Tem Late, (%)	0.093	0.480	0.653
$CD16^{-}$ monocytes, (%)	0.070	0.547	0.716
B cells, (%)	-0.061	0.572	0.720
CD8 Naive, (%)	-0.100	0.662	0.804
Plasmablasts, (%)	-0.037	0.694	0.814
Regulatory T cells, (%)	-0.041	0.726	0.823
CD8 TemRA Early, (%)	-0.037	0.728	0.798
NK $CD56^{DIM}$, (%)	0.016	0.825	0.877
NK $CD56^{BRIGHT}$, (%)	-0.024	0.825	0.850
CD4 TemRA, (%)	0.004	0.893	0.893

Note:

The (Spearman) associations are ordered by p value, with the lowest p values shown at the top. Associations that were selected with a FDR lower than 15% are shown in bold

* False Discovery Rate (estimated)

Figures

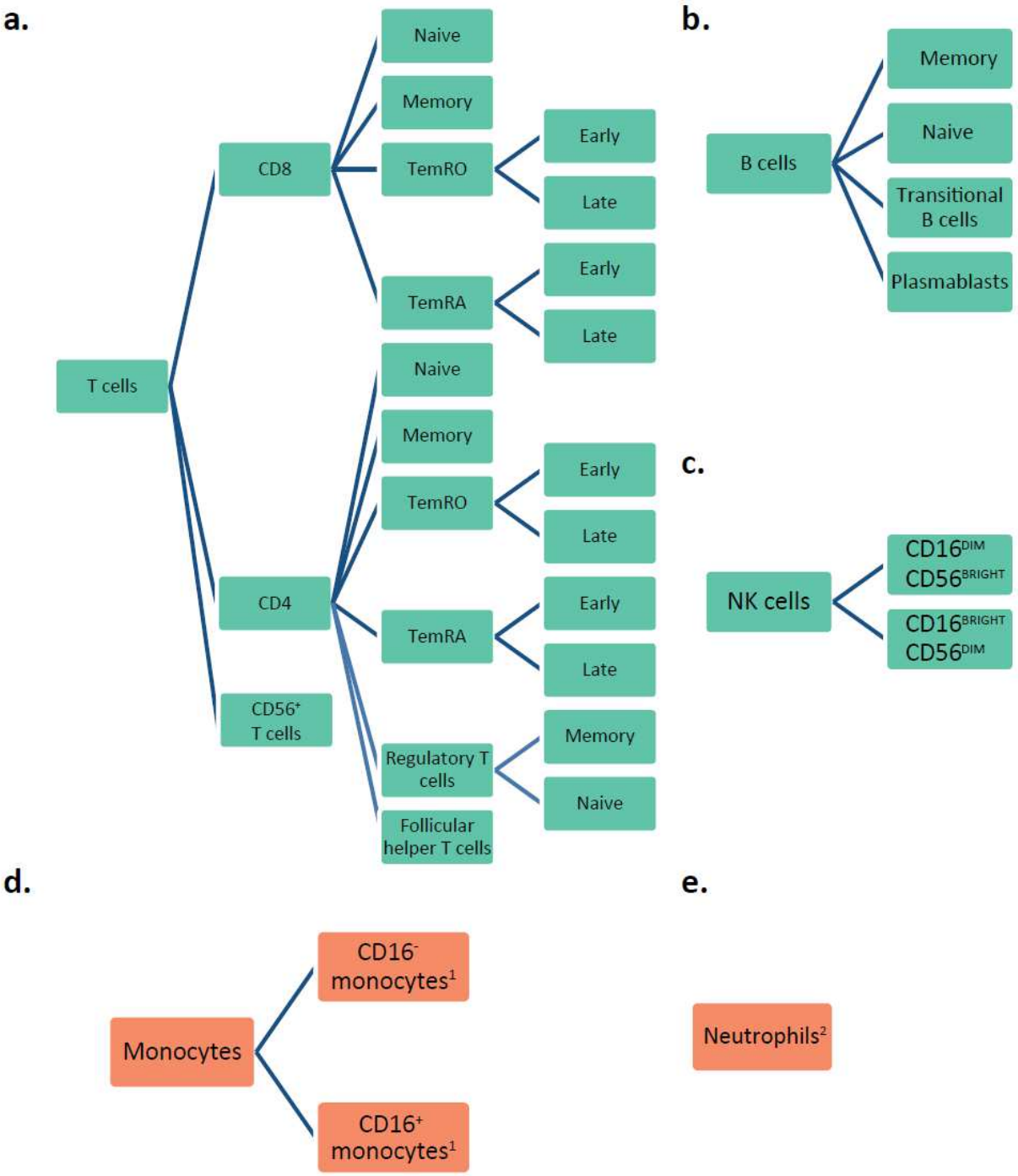

Figure 1

Subpopulations of (a) T cells, (b) B cells, (c) NK cells, (d) Monocytes, and (e) Neutrophils, of which absolute cell numbers were determined. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. See Table S1 for definition of all phenotypes. 1Additionally analyzed on expression of CD38 and HLA-DR. 2Additionally analyzed on expression of CD16.

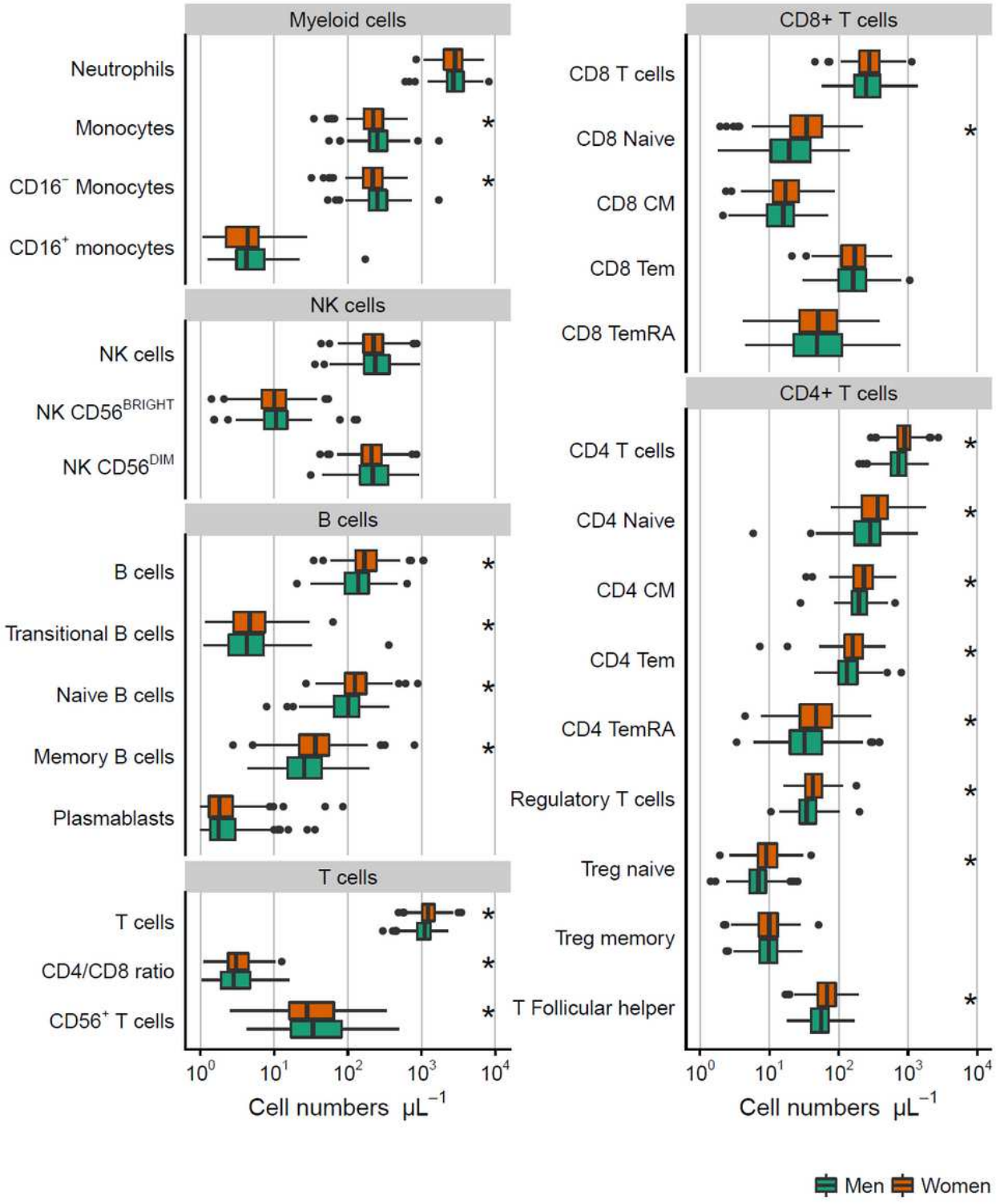

Figure 2

The numbers of cells per leukocyte subpopulations in men (n=145) and women (n=144) above 60 years of age. The boxplots show median values with interquartile range. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. *: Outcomes of tests of the association between leukocyte numbers and sex

(adjusted for age and CMV serostatus) which passed the Benjamini-Hochberg method at a false discovery rate of 15%.

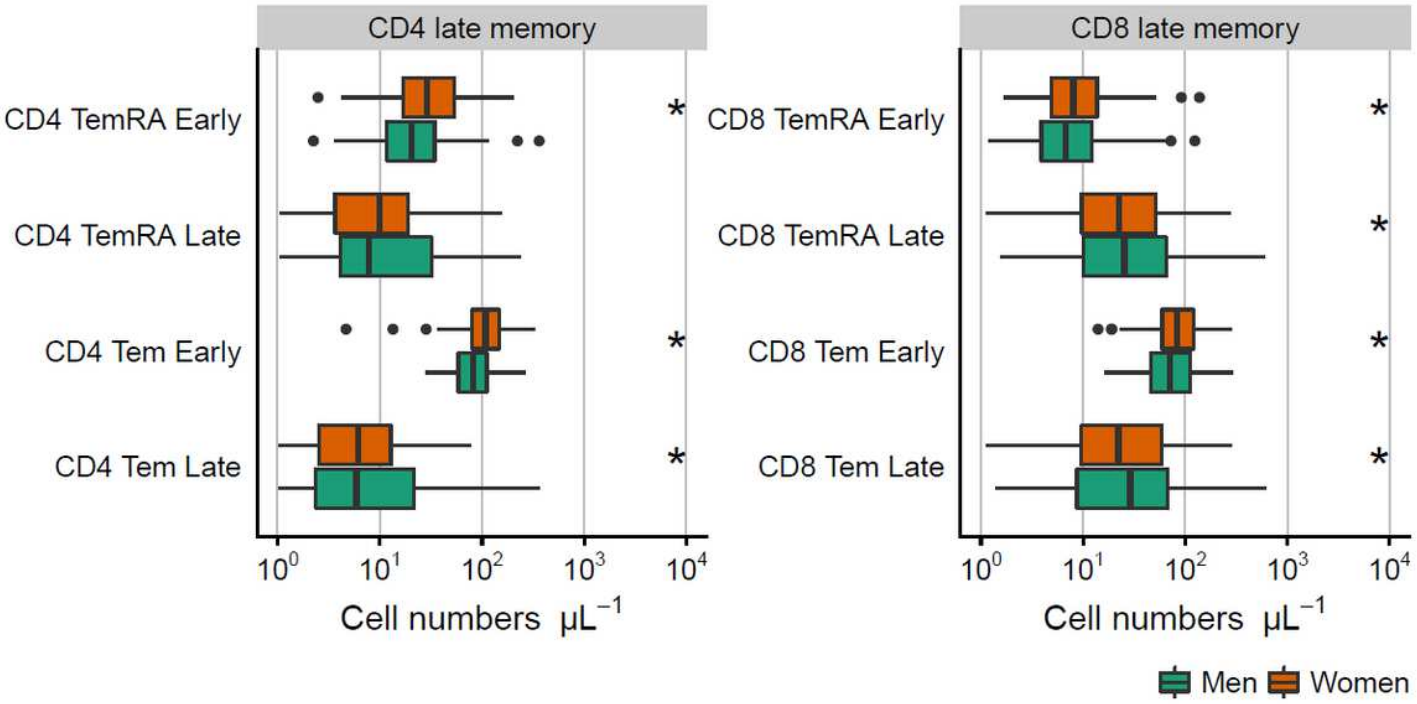

Figure 3

The numbers of CD4 and CD8 Tem and TemRA cells in men (n=145) and women (n=144) above 60 years of age. The boxplots show median values with interquartile range. CM: central memory (CD4 or CD8 positive) T cells. Tem: effector memory (CD4 or CD8 positive) T cells. TemRA: effector memory T cells re-expressing CD45RA. *: Outcomes of tests of the association between leukocyte numbers and sex (adjusted for age and CMV serostatus) which passed the Benjamini-Hochberg method at a false discovery rate of 15%.

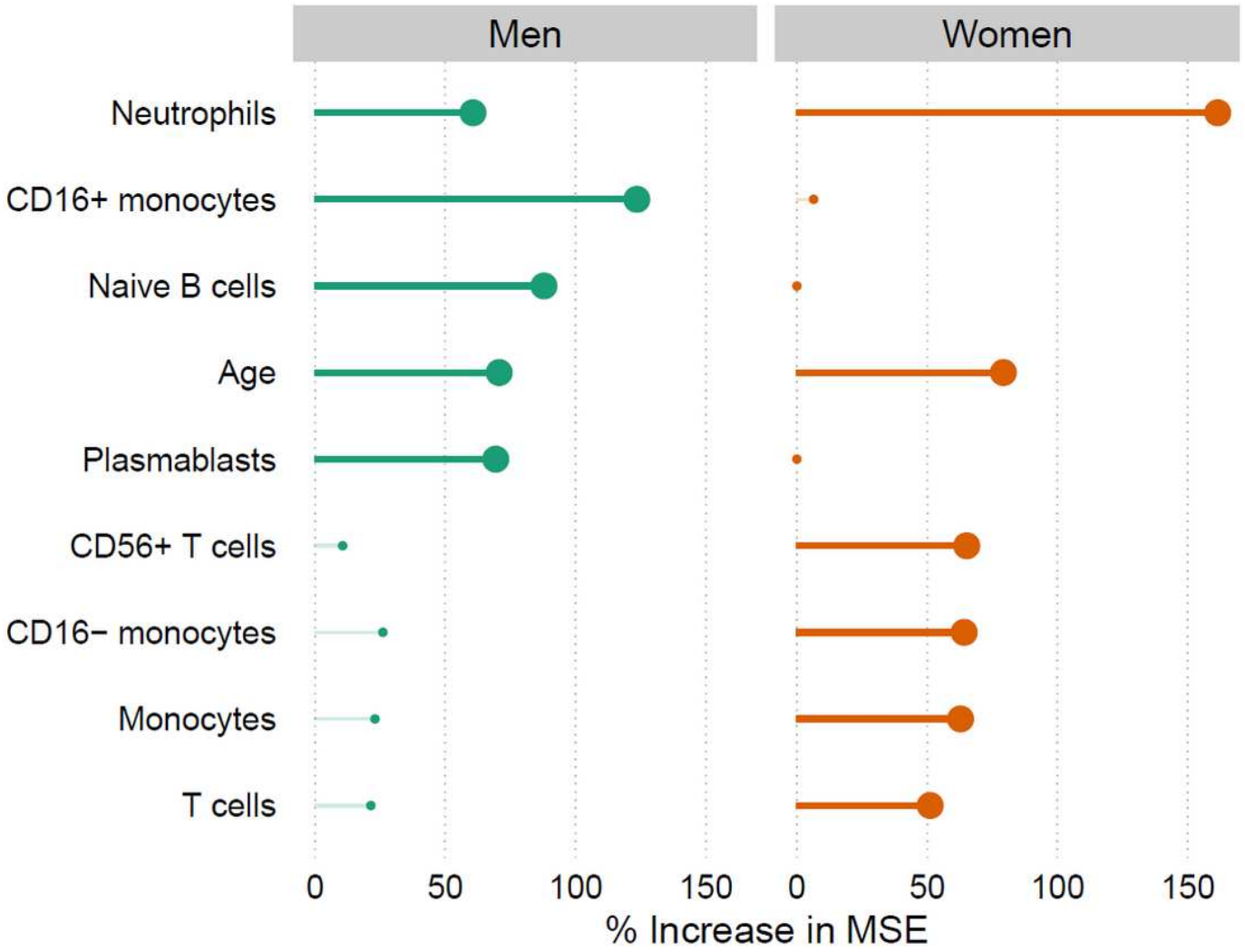

Figure 4

Variable importance plot showing the leukocyte subpopulations and other variables that are most helpful in predicting frailty in men (n=140) and women (n=137). Participants with missing frailty index score data (n=12) were excluded from analysis. The bold lines and dots represent the most important variables that show an increase in mean squared error (MSE) of >50%. See text for the meaning of variable importance.

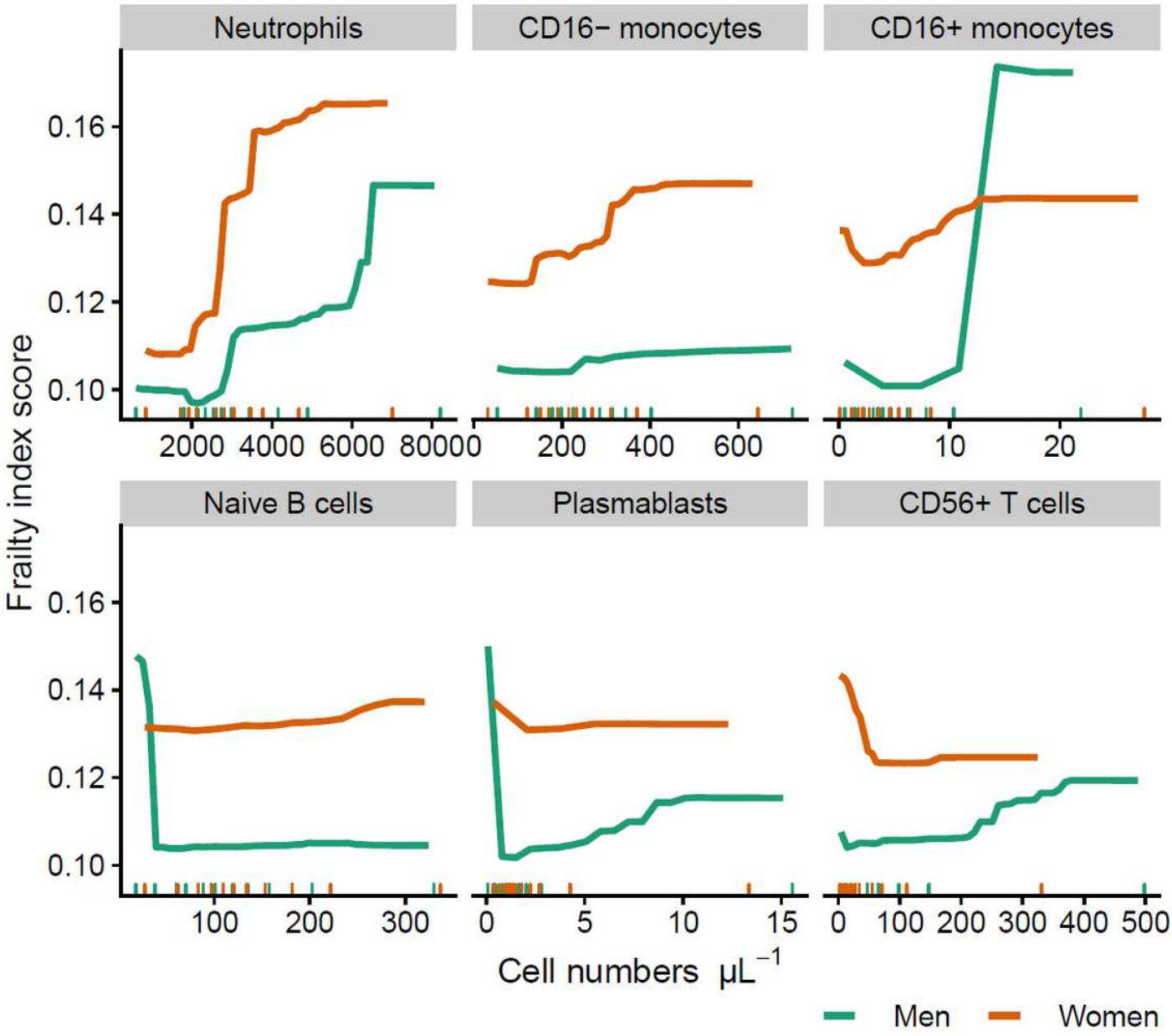

Figure 5

Partial dependence plots illustrating the role of the most important leukocyte phenotypes in predicting frailty in a random forest predictor for men (n=140) and women (n=137). Participants with missing frailty index score data (n=12) were excluded from analysis. The short vertical segments on the horizontal axis represent the deciles of the cell numbers in the data. Range of the figures is restricted to the part containing most of the data.

Supplementary Files

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

- FigureS3gatingothercellsV2.pdf
- FigureS1gatinggeneralV3.pdf
- FigureS2gatingTcellsV2.pdf