Cellular immune phenotypes and worsening scores of frailty-associated parameters over an 18-month period in the very old

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Abstract

Frailty has been related to inflammaging and certain immune parameters. In previous analyses of participants >80 years of age in the longitudinal BELFRAIL cohort study the main focus was on T-cell phenotypes and the association with CMV-serostatus and survival, finding that a CD4:CD8 ratio >5 was associated with frailty, impaired activities of daily living (ADL), and mortality (but only in women). Here, we phenotyped peripheral blood immune cells via multicolor flow cytometry and correlated these with the dynamics of changes in ADL, geriatric depression score, mini-mental state examination, and short physical performance battery from baseline values over 18 months´ follow-up. We found that higher frequencies of B-cells and late-differentiated CD8+ T-cells at 18 months from baseline were associated with ADL impairment that had worsened over the preceding 18 months. There were no significant associations with monocyte, dendritic cell or NK-cell phenotypes. No associations with the Geriatric Depression Scale GDS, the mini-mental state examination, MMSE or the short physical performance battery SPPB were found. Thus, while these results do not establish causality, they suggest that certain adaptive immune, but not innate immune, parameters are associated with a worsened ADL in the very old.

Keywords

Human Aging, T-cells, immunosenescence, longitudinal study, CMV

Introduction

Two major characteristics of human ageing are changes to immunity, termed "immunosenescence" and associated with negative health outcomes, and chronic systemic low-grade inflammation termed "inflammaging". Both may contribute to a vicious circle in which chronic antigen exposure, a somatic cell senescence-associated secretory phenotype (SASP) and age-related autoimmune predisposition promote inflammaging, which in turn impedes clearance of senescent cells (1, 2). The further these processes progress, the higher the risk for morbidity, mortality and degenerative age-related diseases with an inflammatory component (1). This is reflected in the higher serum levels of inflammatory markers, such as interleukin-6 (IL-6) and C-reactive protein (CRP), in frail older adults, together with an increased number of circulating leukocytes (3). Following physiological age-related thymic involution starting at puberty, reduced generation of naïve immune T cells (2) and accumulations of effector and effector-memory T cells (4) characterizes the adaptive immune system. In addition to changes to adaptive immune T-cells and antibody-producing B-cells, innate immunity is also affected and activated monocytes and macrophages may contribute to chronic inflammation in the frail older person (3). To better understand this network and identify potential targets for future therapies, our study aimed to investigate possible associations of adaptive and innate immune signatures with frailty, extending the work of others who have analyzed the potential of immune phenotypes to predict frailty (5). Many cross-sectional studies have established such associations, but the present study is part of the longitudinal BELFRAIL project to investigate interactions between immunosenescence, CMV, frailty, morbidity, ageing and age-related diseases over time in a cohort of community-dwelling individuals >80 years of age in Belgium (6).

Determining an index of activities of daily living (ADL) is one accepted way to assess functional decline associated with frailty in older adults (7) and in those with age-related

diseases, such as neurodegeneration and Alzheimer's disease (8). Previous results from the BELFRAIL study revealed that CMV seropositivity was positively associated with frailty in the very old (9). Further research in this cohort found that older people with a CD4:CD8 ratio >5 had the highest proportion of impaired individuals and that this impairment of physical function was greater in CMV-seropositive persons in association with their lower frequency of effector memory T-cells that may have reflected decreased capacity to control viral latency, leading to a higher inflammatory potential (10). However, other studies from different countries reported that individuals with an inverted CD4:CD8 ratio, at the other end of the spectrum to these BELFRAIL participants, experienced worse functional impairment, a greater degree of dependence and fragility than controls (11) and had higher all-cause mortality on 2-, 4- and 6-year follow-up (12). Thus, presumably for different reasons, either extremely high or extremely low CD4:8 ratios may reflect more generally compromised homeostasis, impaired immune regulatory processes and decreased reserves associated with increasing frailty and mortality in the very old.

The aim of the current study was to extend the earlier BELFRAIL ADL analysis beyond T-cell phenotypes to include frequencies of other leukocytes, such as NK-cells, NKT-cells, B-cells, dendritic cells and monocytes to present a richer picture of immune signatures in older adults and their correlations with ADL, SPPB, global cognition using MMSE and mood by GDS. Here we report the results of this pilot study aimed at identifying informative immune signatures for further follow-up in future studies. Candidate predictive immune biomarkers of functional deterioration in older adults would also provide a framework for testing such markers of immune ageing for their applicability to younger cohorts and could suggest targets for intervention .

Material and Methods

BELFRAIL cohort

BELFRAIL participants were aged from 80 up to 102 years from three areas of Belgium (n=567), recruited by 29 general practitioners. Severe dementia, palliative care and medical emergency were the only exclusion criteria. The details of the study methods are described in previous publications (6, 9, 13). After informed consent and approval by the Biomedical Ethics Committee of the Medical School of the Université catholique de Louvain of Brussels the participants' clinical background was recorded as well as their current status as determined during different examinations and questionnaires described in an earlier manuscript (13). Part of this assessment was physical performance quantified by activities of daily living (ADL) scores, the short physical performance battery (SPPB), mental function by the mini-mental state examination (MMSE) score and Geriatric Depression Scale (GDS) and recording of co-morbidities (13, 14). We also evaluated frailty based on hand-grip strength, Fried criteria, Puts criteria and the Groeningen Frailty Indicator (GFI) (15, 16). Median Values are recorded in Supplemental Table 1.

In brief, the ADL scores were based on six activities: climbing stairs, walking 5 min outdoors without resting, getting up and sitting down in a chair, dressing and undressing oneself, using own or public transportation, and cutting one's own toenails. Response categories ranged from (1) "No I cannot" to (5) "Yes without difficulty." The total score was assessed by adding together the scores of all activities and ranged between 6 and 30.

In addition to baseline analysis (at T0), we analyzed the immune cell phenotypes and the outcomes 18 months later (follow-up T1) and the survival rate 3.3 years after T1 (T2) (see Supplemental Figure 1 for the timeline) (17). In the present study, a sub-cohort of 264 individuals for whom T-cell phenotypes had been measured were included (10, 17), as well

as 250 individuals for evaluation of frequencies of the main peripheral leukocyte populations in PBMCs isolated and cryopreserved at T1.

CMV IgG titres and serostatus at T0 and T1 were determined by ARCHITECT i4000SR (Abbott Diagnostics, Abbott Park, IL) (9). The specific CMV IgG serostatus was also assessed via a recombinant CMV IgG and IgM immunoblot (Mikrogen, Neuried, Germany) using six different epitopes (IE-1, p150, CM2, p65, gB1, and gB2) (10).

Flow cytometry

To investigate T-cell phenotypes the results of a previous BELFRAIL project were used; the antibodies and protocol applied is described in previous publications (10, 17). In brief, PBMC were collected and stored in a sterile viable state in liquid nitrogen until use. Batches of cells were then thawed, dead cells stained, Fc receptors blocked, and surface marker staining performed to identify common T-cell subsets and phenotypes using a BD LSRII flow cytometer. For each run, an independent biological control (large batch of PBMC from a single healthy donor) was included for normalization of run-to-run variability. In addition, a second antibody staining panel was designed using the same surface marker staining protocol and flow cytometry equipment. The following antibodies (and their antibody registry identifier) were used for the leukocyte panel: CD3-APC (AB_314066), CD11c-PE-Cy7 (AB_389351), CD14-APC-H7 (BD Biosciences), CD16-BV-711 (AB_11219184), CD20-PerCP (AB_1937324), CD45-V500 (AB_1937324), CD56-BV605 (AB_2728700), CD57-PE (AB_2033965), CD123-BV421 (AB_11153668), CD161-FITC, HLA-DR-PerCP-Cy5.5 (AB_394453), ethidium monoazide bromide (EMA, Biotium) to stain dead cells and discriminate from the living cells. Supplemental Figures 2, 3 and 4 show the gating strategies applied using FlowJo Software (Treestar).

Data analysis

The aim of the current study of the longitudinal BELFRAIL cohort was to establish whether immune measures paralleled any changes in participant ADL values from T0 to T1. Participants were stratified depending on whether their ADL values decreased (designated -1), stayed similar (0) or in rare cases increased (1) between T0 and T1. Frequencies of T-cell subsets (at T1) were then compared between these groups. In addition, participants were grouped according to ADL scores higher or lower than median, excluding those with exactly median values, to analyze the relationship between ADL and immune parameters.

Mann Whitney testing was used for comparisons between two groups and the Kruskal-Wallis test for multiple comparisons using GraphPad Prism 5 software (GraphPad, California, USA), which was also used for creating the images. P values <0.05 were considered significant.

For analyzing the relationships between age and B-cell frequencies separately in women and men, univariate linear regression and Spearman tests were performed. To investigate the impact of B-cell frequencies on all-cause mortality, Multivariable Cox regression analysis was performed as in the previous survival study (17).

Results

T cell phenotypes

The aim of the current study was to establish whether certain immune measures at T1 characterized participants whose ADL values had worsened from T0 to T1. Participants were accordingly stratified depending on whether their ADL values decreased (designated -1), stayed similar (0) or in rare cases increased (1) between T0 and T1. Figure 1 shows results for different CD4+ and CD8+ T-cell phenotypes. For CD4+ T-cells, the frequencies of early and

late-differentiation stages at T1 were the same regardless of whether the ADL had changed over time (Fig. 1A-C). For CD8+ T cells, a trend towards higher frequencies of CD8+ T-cells with a more differentiated phenotype and lower frequencies of cells with a naïve phenotype in subjects who had transitioned to a more impaired ADL can be discerned. Thus, frequencies of CD45RA+CCR7+CD27+CD28+ T-cells (naïve) are lower in the ADL -1 group relative to the ADL 0 group (Figure 1D, P=0.0592) and reciprocally, the CD8+ late-differentiated TEMRA cells (CD27-CD28-CCR7-CD45RA+) appear to be somewhat higher (Figure 1E).

Because these results comparing all individuals in the two groups (ADL 0 vs. ADL -1) showed some tendencies but no statistically significant differences and because CMV is known to markedly amplify CD8+ TEMRA cells, the ADL -1 and ADL 0 groups were divided into CMV-seronegative and CMV-seropositive individuals. While for early-differentiated T-cells no significant difference was observed (Figure 2A), the lower frequencies of CD8+ TEMRA cells in ADL 0 vs. ADL -1 individuals was limited to the CMV+ subgroup and not found among the CMV- older adults (Figure 2B). However, this difference also just failed to achieve statistical significance.

In an additional analysis the study participants were divided according to ADL scores less than or above the median (23 for T1) and CMV-serostatus was taken into account. While frequencies of early-differentiated CD8+ T-cells were not different between these groups (Figure 3C), frequencies of late-differentiated CD8+ T-cells tended to be lower in CMV+, but not CMV- older adults with ADL scores higher than the median (p=0.047). When stratifying this group of CMV+ individuals by sex, the effect seemed to be especially pronounced in women, although again not significantly so (Figure 2D).

Note that the ADL values *per se* at timepoint 0 did not impact T-cell phenotypes when the BELFRAIL cohort was divided into those older adults with a median ADL greater and those with a median ADL less than the median of 25 (supplemental table).

Frequencies of CD20+ B-cells

In addition to the analysis of immune phenotypes using T-cell data, frequencies of other leukocytes were also determined and their relation to frailty markers assessed. Of all the immune cell subsets, only cells carrying some B cell markers were significantly different between groups, and no other associations between innate or adaptive immune parameters were informative at T1 for differences between the ADL 0 and ADL -1 groups. Thus, frequencies of CD20+ B-cells were significantly higher in participants with worsened ADL compared to either the ADL 0 or ADL +1 groups (Figure 3). This difference was independent of whether the whole CD45+ leukocyte cell population was used as the reference in flow cytometry (p=0.0097) or whether B-cell frequencies within the CD14- morphological lymphocyte gates (p=0.0060) were determined (Supplemental Table2).

In addition to ADL, other parameters informative for frailty were also characterized and values at T0 and T1 compared. Study participants were categorized according to their worsening or improvement as measured by the short physical performance battery SPPB, geriatric depression scale GDS15 and mini mental state examination MMSE. However, no significant differences were observed in B-cell frequencies between any of the groups (Figure 3).

To analyze potential effects of other pathologies, study participants were grouped according to diseases at baseline. There were positive associations between thyroid dysfunction and higher B cell frequencies (p=0.04), and myocardial infarction with lower B cell frequencies (p=0.03). No associations were seen with an extensive range of other pathologies, including

anemia, COPD, arthrosis, osteoporosis, depression, hip prosthesis, hypertension, hyperlipidemia, diabetes, angina pectoris, cardiomyopathy, transient ischemic attack peripheral artery disease, cerebrovascular accident, decompensated heart failure, episode AF, known valvular disease or peripheral edema anamnesis. Less than 16 participants were affected regarding asthma, Parkinson´s, arthritis, cancer, renal insufficiency and knee prosthesis and so these pathologies were not analyzed. When participants were grouped according to having at least one new pathology between T0 and T1, or reported hospitalization, no significant differences in B-cell frequencies were seen.

As additional potential confounders age (supplemental Figure 5) and CMV infection status (Supplemental Figure 6) were considered, but also here no impact was found. A linear regression analysis and a Spearman test did not reveal any association between frequencies of CD20+ cells and age either in men or women - at least for the small age range of 81 to 97 years studied here, which for immune ageing might represent a plateau phase of longevity.

In a detailed serological CMV analysis, we also assessed semi-quantitative anti CMV IgG titre changes between T0 and T1 (T1/T0 ratio) (Supplemental Figure 6A). In addition, a detailed IgG (Supplemental Figure 6B) and IgM (Supplemental Figure 5C) serological specificity analysis for 6 prominent CMV proteins (p65, p150, IE1, CM2, gB1 and gB2) was undertaken, but also revealed no relationships between CMV and B-cell frequencies. The same was true for IgG avidity (Supplemental Figure 5C). Multivariable Cox regression survival analyses in men and women separately also showed no significant differences in B-cell frequencies (data not shown). Analysis based on the outcome of individual age-associated pathologies compounded to a single pathology score also failed to reveal a significant impact on frequencies of B-cells (Supplemental Figure 7).

Phenotypes of other leukocytes

Frequencies of other leukocytes, such as NKT-cells (CD3+CD56low), NK-cells, lineage-negative, HLA-DR+ dendritic cells, and monocytes did not differ significantly at T1 between participants whose ADL had worsened or remained stable or even improved (Supplemental Table). A more detailed analysis of NK-cell differentiation via quantifying the level of expression of CD16, CD56 and CD57, or dendritic cells (pDC, mDC, characterized by CD123/CD11c expression) or monocyte (classical, intermediate, non-conventional) subsets also failed to reveal any differences. In addition to differentiation phenotypes of NK-cells from CD56high CD57- to CD56low CD57- to CD56low CD57+ also the expression of CD161 on NK-cells was studied, but no associations with ADL were found. No associations of CD16+ proinflammatory mDCs and ADL change were observed, but there was a slight trend for lower frequencies in study participants with ADL values greater than the median of 25. Finally, populations of myeloid-derived suppressor cells (MDSC), defined as CD20-CD56-CD3-CD14-HLA-DR- and CD20-CD56-CD3-CD14-HLA-DR- cells were a little higher in participants with higher than median ADL scores.

Discussion

Previous investigations showed a link between immune signatures and frailty and so this BELFRAIL study aimed to validate these findings, generalize them and further extend them by adding additional parameters. Here, as well as T-cell frequencies, also B cells and innate immune cell subsets were examined, but no associations with most of the scores of impaired health in older adults were found except for higher B-cell frequencies in participants whose ADL had worsened between T0 and T1. Study design and materials and data availability imposed severe limitations on this study, but its strength is its longitudinal nature (health

assessment at two time points) and the large database on health-related conditions. Thus, we employed deep phenotyping to seek peripheral cellular immune parameters distinguishing between participants who had worsening ADL values over the preceding 18-months and those whose ADL had remained stable or even (very rarely) improved. Because of the large inter-individual differences between people in this advanced age group, correlations with immune phenotypes rarely achieved statistical significance. However, certain trends in T and B cell distribution were noted between those individuals who had suffered worsening ADL and those who had not. These differences were not present in a wide range of other health parameter assessments and were not affected by most of the extensive range of pathologies recorded for these very old people. This emphasizes the potential associations between immune parameters and the ADL assessment of health status, but not the other health assessments. However, group associations even with ADL values were very weak and one must conclude that immunity as assessed in the manner reported here has little impact on worsening health status.

There are many studies on immune system alterations (18) and frailty, for example, Valdiglesias et al. investigated this connection by determining the frequencies of lymphocyte subsets in older adults in a search for potential biomarkers for frailty and associated pathologies (19). After establishing reference values and characterizing confounding age and gender effects on T-cell and B-cell populations they reported no significant differences between non-frail and prefrail individuals (19). In a subsequent study they observed a higher CD4:CD8 T-cell ratio and a lower frequency of CD19+ cells in study participants with reduced physical activity, and also pointed to a connection with cognitive impairment (18).

The importance of the confounders can be stressed by proposing ageing as a disease using the disease criteria of the international classification of diseases (here ICD-11) (20, 21). In addition, a previous study found that frail women had an increased risk of developing

"instrumental ADL disability, institutionalization, and death, independently of multiple potentially confounding factors" (22). Another study reported a connection between immune parameters and physical disability in extremely long-lived women (23). Thus, our null hypothesis for this extensive survey in the BELFRAIL cohort was that we would see extensive correlations between multiple health parameters centered on but not limited to ADL values and immune signatures, and that these would change over time on follow-up. In both of these expectations we were disappointed.

The strength of this work is in the longitudinal nature of the BELFRAIL study, making it possible to correlate changes in ADL status over 18 months in older individuals with their peripheral blood immune signatures at that time. Thus, following up on earlier results, in the current study a group of participants of the BELFRAIL study were analyzed for ADL performance at two time points and a potential connection to immune ageing investigated, taking into account these potential confounders. Because CMV can also be considered a confounder (24), it was planned to include this variable in the analysis. However, the number of CMV-seropositive study participants was similar in the ADL cohorts studied, and most of the older adults were CMV+, so the groups were not further divided into CMV- and CMV+ individuals.

In contrast to the Spanish study, we were not able to confirm a connection between low B-cell frequencies and frailty-associated ADL worsening, but on the contrary, we found higher frequencies of B cells (although we did use CD20 instead of CD19 to identify the B cells). In our case the marked B-cell population effect was observed in analysis of changes of ADL values over time. Other physical activity-associated parameters, such as SPPB and GDS15 were not related to B-cell frequencies. Limiting comparability, the Validiglesias et al. studies were based on a cohort of older adults with a greater age range of 65 to 95 years than the present study and B-cell levels can be different between older adults under and over 85 years

of age, as previous studies showed (18, 19, 25). A possible explanation for this is that at some point individuals may have reached an immune ageing end point. As a side observation, Validiglesias et al. found higher B-cell levels in women compared to men, and we could confirm this for CMV- (non-significant) and CMV+ individuals (p=0.0010 for CD20+ within CD45+ cells and p=0.0009 for CD20+ cells within lymphocytes, data not shown). Another study highlighting the impact of sex on B-cells revealed a shift from CD27+ IgD+ and CD27- IgD+ B-cells to a more late differentiated CD27-IgD- B-cells in nonagenarians compared to controls in women but not in men. On the other hand, the number of CD27-IgD- B-cells correlated with inflammation determined by IL-6 levels, physical performance assessed by the Barthel index and frailty scores only in men (26). This further strengthens the relevance of not only analyzing total B-cells, but also their phenotypes, the lack of which is a limitation of our study.

Not only B-cells, but also NK-cells have been related to frailty in published studies. Levels of IL-15, reported as a key factor in NK-cell development and survival, were reported to decrease with age and this was considered a possible frailty pathway (27). Nevertheless, little influence of the ADL was seen on the frequencies of NK-cells and other innate cells in the current study (Supplemental Table 2). IL-15 is also reported to regulate memory and effector CD8-Tcells associated with longevity and protection against viral re-infections (28).

To monitor ageing and find biomarkers for those who deviate into frailty, considered as an indicator for higher risk of institutionalization (29) and linked to multi-morbidity (especially in women) (30), the present study identifies relatively few peripheral blood cellular immune parameters informative for worsening of ADL scores over an 18-month period in very old Belgians. Data from T2, a 3-year follow-up, would have helped to reveal additional potential correlates and identify factors on which to focus when discussing treatments for immunosenescence to promote healthy ageing, but this remains to be accomplished.

Conflicts of interest

All authors declare no conflict of interest.

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BV, GvP, JMF, CM planned and conducted the BELFRAIL study and its medical assessments, and contributed to the manuscript writing. WA collected blood samples, isolated PBMCs, performed the T-cell flow cytometry analysis and helped with the manuscript, KH performed the detailed CMV serology, LO analyzed the leukocyte phenotypes by flow cytometry, DG developed the leukocyte flow cytometry panel, performed statistical analysis and contributed to writing the manuscript, GP supervised the immunological analysis and contributed to writing the manuscript.

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Figure 1: Early (A, D), and late differentiated (B, C, E, F) CD4+ (A-C) and CD8+ T-cells (D-F) at T1 in relation to ADL worsening (-1), remaining the same (0) or improving (1) between T0 and T1 in the BELFRAIL study. Bars represent median values. Groups were compared with the Kruskal Wallis test (upper P values). Below the X-axis the p values are given after Mann Whitney testing to compare individuals with -1 (deterioration) and 0 (no difference over time) of ADL, excluding those with improving ADL (too few).

Figure 2: Frequencies of early (A), and late differentiated (B) CD8+ T-cells (F) in relation to ADL worsening (-1) or remaining the same (0) in CMV-seronegative or CMV-seropositive individuals at T1. Frequencies of early (C) and late (D) CD8+ T-cells from CMV- or CMV+ study participants (at T1 including seroconverters in the CMV+ group) with ADL scores above or below the median values at T0 (median 25) and T1 (median 23). Participants with median ADL scores were excluded as well as those without available ADL values or CMV serostatus at a given time point. On the right, CD8+ T-cell frequencies of CMV+ individuals at T1 are displayed with the subgroup divided by gender and considering sex-specific ADL mean scores of 26 (all of the men) and 22 (all women). Bars represent median values. Groups were compared with the Mann Whitney test.

Figure 3: Impact of age-related clinical parameters on CD20+ B-cell frequencies. Study participants were grouped according to ADL impairment (-1), constant (0) and improvement (1) between timepoints T0 and T1 regarding results of SPPB = short physical performance battery, GDS15 = geriatric depression scale, MMSE = mini mental state examination and ADL = activities of daily living. The median values are represented by a bar. Kruskal Wallis analysis: p = 0.0097 for ADL. Mann-Whitney comparison between -1 and 0, P = 0.0058.

Figure 1

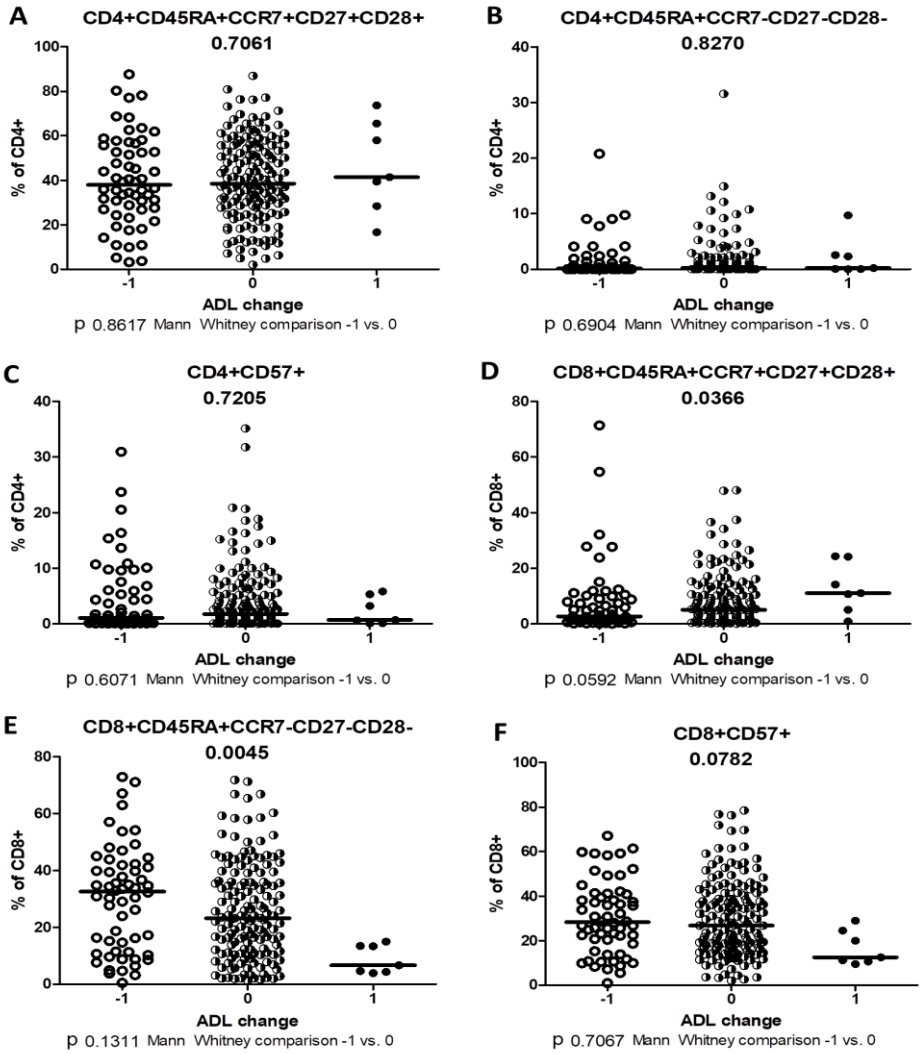

Figure 2

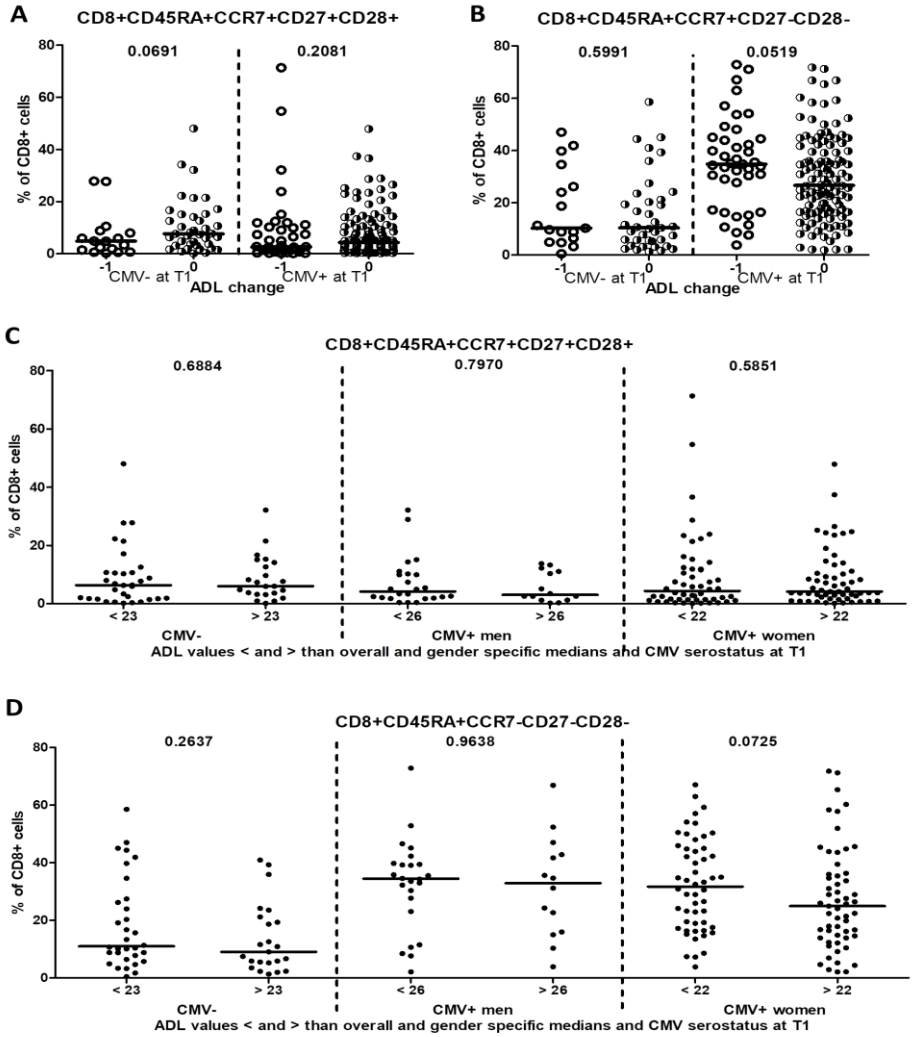

Figure 3