Author's response to reviews

Title: Immunosenescence in the Nursing Home Elderly

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Dear Editors,

Thank you for giving us the opportunity to revise and resubmit our manuscript entitled, “Immunosenescence in the Nursing Home Elderly” (MS ID# 1050835238118282 R1). Our responses to the Reviewers’ suggestions are as follows:

**Reviewer #1**

1. *The authors indicate that “CD4+ and CD8+ immune phenotypes and T-regs were expressed as a percentage of CD3+ and NK cell subsets were expressed as a percentage of CD3- cells”. Whereas these values might be correct for the T cell subsets, it is very unlikely that the median of mature NK cells are 12.1 (IQR 7.9 – 16.6) within the CD3- populations. On the contrary the values presented are more likely referred to the total gate of PBLs. Please check and revise the calculations of these percentages.*

   The Reviewer is correct and we regret our oversight. All immune phenotypes including CD4+, CD8+, T-regs and NK cells are expressed as a percentage of total lymphocytes. We have revised the manuscript Methods section to read as follows:

   “CD4+ and CD8+ immune phenotypes, T-regs and NK cell subsets were expressed as a percentage of total lymphocytes” (Page 7, Line 14).

2. *Please indicate the statistical analysis used in the different tables.*

   The tests of significance have been added to Table 2 and Table 3 in the revised manuscript.

3. *The authors discuss the low frequency of old individuals with ratios CD4/CD8 <1 (6,5%) compared with the frequency of IRP in the OCTO study (14%). Please consider the possibility to include studies of other groups (Ferrando-Martinez et al., 2013) that have also found frequency of CD4/CD8 <1 in approximately 7% of old individuals. The age range can also be an important factor as the frequency of donors with CD4/CD8 <1 is lower in the NONA cohort and disappears in centenarians.*

   We thank the Reviewer for drawing our attention to these references. We have included these two references in our manuscript (References #8 and #30) and added the following sentences to our Discussion section:

   “In a Spanish study that enrolled 151 elderly people ≥65 years only 7.9% of the cohort had a CD4+/CD8+ T-cell ratio <1.0 [30].” (Page 13, Line 2)
“The NONA cohort has also demonstrated that at very advanced ages (>90 years), it is possible to move from having a CD4+/CD8+ T-cell ratio <1.0 to above 1.0, which is another possible reason why our study had so few people with CD4+/CD8+ T-cell ratios <1.0 [8].” (Page 13, Line 13)

4. **The authors should consider including in the discussion the possibility that the changes observed in T and NK cells are the consequence of a remodelling of these subpopulations due decreased output of new (naïve T or immature NK cells) and the expansion of effector or senescent cells associated with chronic antigenic stress (e.g. CMV).**

We agree that immune phenotype findings in the elderly could be due to remodeling and have now stated this explicitly in our Discussion section as follows:

“These differences are consistent with existing dogma, that the remodelling of the T-cell compartment seen with advanced age is due to a decrease in naïve T-cells due to thymic involution and an increase in terminally differentiated T-cells, possibly due to chronic antigenic stimulation from CMV [1].” (Page 11, Line 9)

and

“We speculate that the higher numbers of mature NK cells could be due to the same mechanism leading to higher numbers of memory T-cells in the elderly; chronic antigenic stimulation [1].” (Page 14, Line 8)

5. **The authors define senescent NK cells as CD56dimCD16negative (18). However other authors have recently shown that the expression of CD57 is a marker of highly differentiated NK cells. Are there evidences of a relationship between these 2 parameters?**

The reviewer raises an interesting point regarding the relationship between CD57 expression and CD16 expression CD56(dim) NK cells. However, we did not measure CD57 expression in these experiments and cannot speculate on the relationship between the CD16- CD56(dim) cells and CD57+CD56(dim) cells.

**Reviewer #2**

1. **The paper might be interesting, however the conclusions cannot be accepted because it is not possible to compare 262 cases versus 16 controls.**

We acknowledge that the small numbers of controls is a limitation of our study and had previously stated this in our Discussion. The small control group increases the possibility of a Type II error – not finding a difference between the two groups when one exists. However, our control group size is similar to at least 4 other similar studies, and our findings are comparable to studies involving
community dwelling elderly, increasing the confidence in our results. Our Discussion section previously read as follows:

“Although our study has many strengths including a large sample size of nursing home residents and detailed clinical information including frailty and nutritional status as well as comprehensive immune phenotyping, limitations of our study include the small size of the comparator group.”

We have substantially expanded our limitations section in the Discussion section to read as follows:

“Although our study has many strengths including a large sample size of nursing home residents and detailed clinical information including frailty and nutritional status as well as comprehensive immune phenotyping, limitations of our study include the small size of the comparator group, which may have increased our risk of not finding a difference between the two groups when one exists (type II error). However our control group size was comparable to at least four other similar studies where the control group size ranged from 15 to 21 adults [3, 36-38] and our findings were similar to studies involving community dwelling elderly, increasing the confidence in our results. (Page 14, Line 14).

2. In addition, it should be important to know the CMV status of the cases, due to the differences observed when compared to literature data.

We have now analyzed the T-cell immunity to CMV by flow cytometry. We added the following paragraphs to our Methods, Results and Discussion sections:

“PBMCs were isolated and frozen using a validated common standard operating procedure [20]. PBMCs were thawed and CMV-reactive T-cells were identified by stimulating PBMCs with a pool of overlapping peptides spanning the immunodominant pp65 protein of CMV (PepTivator pp65, Miltenyi Biotec) according to our published protocols [21]. Briefly, thawed PBMCs were cultured overnight at 37°C and stimulated with CMV peptides (2 ug/ml) for 1 hr at 37°C. A matched set of PBMCs were stimulated with DMSO as a negative control. Brefeldin A (BD Biosciences) was then added according to the manufacturer’s instructions and the cells were incubated for an additional 4 hours. The cells were stained with anti-CD4-PacificBlue and anti-CD8-AlexaFluor700, permeabilized and finally stained with anti-IFN-γ-APC, anti-TNF-α-FITC and anti-CD3-QDot605. CMV-reactive T-cells were identified as CD3+ (CD4+ or CD8+) IFN-γ+ TNF-α+.” (Methods section, Page 7, Line 21)

“Of the 262 nursing home elderly enrolled in the study, 242 had PBMCs available for assessment of T-cell immunity to CMV. In total, 217/242 (90%) of individuals had evidence of prior CMV infection.” (Results section, Page 10, Line 21)
“Indeed, 90% of the elderly nursing home residents in our study had evidence of CMV immunity, which is consistent with prior studies where infection with CMV is estimated to be prevalent in >85% of those aged over 80 years [23].”

(Discussion section, Page 11, Line 12)

3. Otherwise, in my opinion, it is not correct to write, “expected to die within 30 days.” It should be better to write, “affected by serious diseases.”

We have changed the wording of our exclusion criteria from “expected to die within 30 days” to “participants affected by serious diseases with very poor short-term prognosis” (Page 5, Line 22).

Respectfully submitted,

Jennie Johnstone, on behalf of all the study authors.