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Immune defects associated with lower SARS-CoV-2 BNT162b2 mRNA vaccine response in aged people

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Conflict-of-interest policy

The authors have declared that no conflict of interest exists.
ABSTRACT

The immune factors associated with impaired SARS-CoV-2 vaccine response in the elderly are mostly unknown. We studied >60 and <60 years old people vaccinated with SARS-CoV-2 BNT162b2 mRNA before and after the first and second dose. Aging was associated with a lower anti-RBD IgG levels and a decreased magnitude and polyfunctionality of SARS-CoV-2 specific T cell response. The dramatic decrease in thymic function in aged people with >60 years of age, which fueled alteration in T cell homeostasis, and lower CD161+ T cell levels were associated with decreased T cell response two months after vaccination. Additionally, a deficient dendritic cell (DC) homing, activation and Toll like receptor (TLR)-mediated function, along with a proinflammatory functional profile in monocytes, were observed in the >60 years old group, which was also related to lower specific T cell response after vaccination. These findings might be relevant for the improvement of the current vaccination strategies and for the development of new vaccine prototypes.
INTRODUCTION

Immune aging is sustained by multifaceted remodeling of the innate and adaptive immunity, which includes a diminished response to new antigens, a decreased memory T cell response and a persistent chronic inflammation (1–4). In consequence, immune aging leads to more severe consequences of viral infections as well as lower protection following vaccination (5). Severe acute syndrome coronavirus 2 (SARS-CoV-2) infection and its associated disease, COVID-19, are known to have a higher impact in aged people. In fact, delayed viral clearance, prolonged disease and higher COVID-19 fatality rate have been related to age (6) and approximately 80% of hospitalizations involved people older than 65 years (7, 8).

Vaccination is the most effective tool for the prevention of the serious symptomatology caused by SARS-CoV-2 and other viral infections, especially for vulnerable populations as elderly people (9, 10). BNT162b2 mRNA vaccine, commonly known as Biontech/Pfizer vaccine, has shown high safety and efficacy against severe outcomes of COVID-19 (11). The two-dose vaccination of this SARS-CoV-2 vaccine induces a strong humoral response measured by the magnitude of binding antibodies to coronavirus Spike (S) protein and the neutralization capacity of the antibodies (11, 12). In addition, notable SARS-CoV-2 S-specific CD4+ and CD8+ T cell responses have been observed after BNT162b2 vaccination (13–15).

In spite of the promising results of the BNT162b2 vaccination, a lower effectiveness, in terms of COVID-19 symptoms, admissions to hospital and deaths after the vaccination, has been reported in elderly people (16–18). Moreover, other studies have described
lower levels of neutralizing antibodies in vaccinated elderly comparing with younger subjects (19–21), especially six months after the second dose (22, 23). In addition, low levels of S-specific T cell response after vaccination have been shown in the elderly (20). However, the adaptive and innate immune factors associated with the lower vaccine response in elderly people are not yet characterized.

A better understanding of the age-related immune dysfunction to SARS-CoV-2 vaccine is crucial for future vaccination strategies to improve older adults’ protection against this virus. Thus, the aim of the study was to investigate the major immune alterations in aged people, in terms of both SARS-CoV-2 specific adaptive and innate immunity, associated to a lower response to the SARS-CoV-2 BNT162b2 mRNA vaccine.
RESULTS

Association of SARS-CoV-2 specific IgG levels with age

In this study, we included 54 healthy adults vaccinated with BNT162b2 mRNA vaccine against SARS-CoV-2, classified according to their age: 33 young people with <60 years of age (median, 29 years [interquartile range, IQR 26-49]) and 21 aged people with >60 years of age (median 73 years [72-74]) (Figure S1). Comorbidities of all donors are presented in Figure S1. As it was expected, the aged group showed a higher percentage of donors with comorbidities, being the predominant ones cardiovascular diseases and arthrosis (Figure S1). Three subjects were excluded from the study due to a positive result for SARS-CoV-2 RNA PCR or antibodies against receptor-binding domain (RBD) of the S protein of SARS-CoV-2 prior to vaccination. The innate and adaptive immunity parameters were analyzed before vaccination (PRE), three weeks after the first dose, just before the administration of the second dose (1D) and two months after the second dose (2D) (Figure S1).

Firstly, anti-RBD SARS-CoV-2 IgG levels were quantified by RBD-specific enzyme linked immunosorbent assay (ELISA) in >60 years old and <60 years old people at the three time points described above. In accordance to previous studies (11, 20), the BNT162b2 mRNA vaccine induced the production of SARS-CoV-2 specific IgG levels, being these levels much higher after the second dose comparing with the first dose (Figure 1A). Although no significant differences were observed between aged and young subjects, young people tended to produce higher levels of specific antibodies after the first dose (Figure 1A). This tendency was also observed when we correlated anti-RBD IgG levels and age after the administration of the first dose (Figure 1B, left) and after the second
one (Figure 1B, right). In addition to SARS-CoV-2-specific antibodies, we also determined IgG autoantibodies against IFN-α in plasma of the studied donors. In fact, anti-IFN-Is have been previously observed in severe COVID-19 patients (24), but the role of these antibodies in the context of SARS-CoV-2 vaccination remains unknown. Anti-IFN-α IgGs were observed only in two donors; these autoantibodies were found before and after SARS-CoV-2 vaccination (Figure S2). Therefore, only two donors were positive for anti-IFN-α autoantibodies and this was not related to the response to SARS-CoV-2 vaccination.

**Aged people show a lower and less polyfunctional SARS-CoV-2 Spike-specific CD4+ and CD8+ T cell response after vaccination**

We investigated the magnitude and polyfunctionality of SARS-CoV-2 S-specific CD4+ and CD8+ T cell response through intracellular cytokine staining. We analyzed these parameters in total memory (Memory), central memory (CM), effector memory (EM) and terminal differentiated effector memory (TEMRA) CD4+ and CD8+ T cells (Figure S2A). Three weeks after the administration of the first dose, CD4+ T cells principally produced interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) as an acute response to SARS-CoV-2 S protein, but also expressed low levels of the degranulation marker CD107a, perforin (PRF) and interleukin (IL)-2 (Figure S2B). Importantly, CD4+ T cells from people with >60 years of age showed a lower SARS-CoV-2 S-specific IFN-γ production and cytotoxic response, reflected in the percentage of CD107a+ and PRF+ cells, after the first dose of vaccination but mainly after the second dose (Figure 2A). In fact, the second dose of vaccination induced an increase in
the cytotoxic function by CD4+ T cells in <60 years old people but not in >60 years old ones (Figure 2A, Figure S2C). Regarding CD8+, as it was observed in CD4+ T cells, aged people showed a lower SARS-CoV-2 S-specific CD8+ T cell response, based mainly on the production of IFN-γ and the cytotoxic capacity (CD107a+ and PRF+), two months after the second dose (Figure 2B).

To determine the quality of the specific T cell response to SARS-CoV-2 vaccine, we analyzed the polyfunctionality of CD4+ and CD8+ T cells, which is defined by those cells that simultaneously produce multiple cytokines and degranulate (functions). In general, a low polyfunctional T cell response was observed after the vaccination with BNT162b2 mRNA vaccine in both aged and young participants (Figure 2C). However, a more polyfunctional memory CD4+ T cell response to SARS-CoV-2 was observed in <60 years old people comparing with >60 years old ones after the first dose and a trend after the second dose (Figure 2C). The polyfunctional profile of the rest of CD4+ T cell subsets showed a similar pattern, with the exception of TEMRA CD4+ T cells that presented higher polyfunctionality after the second dose in young people (Figure S2D).

To further characterize this SARS-CoV-2-specific T cell response to vaccination, we analyzed different combinations of the studied functions. CD4+ T cells expressing CD107a and PRF simultaneously were enriched in young people with <60 years of age two months after the second dose of vaccination, confirming the higher T-cell cytotoxic capacity of young people (Figure 2C and 2D). Combinations including both IFN-γ and TNF-α and the ones including only IFN-γ were mainly observed in young people after the first and second dose of vaccination (Figure 2D). Furthermore, the percentages of T cells expressing three functions at the same time (e.g. IFN-γ+ CD107a+ PRF+ or IFN-γ+ IL-2+ TNF-α+) and other two-function combinations (e.g. IFN-
γ+ IL-2+) were also higher in the <60 years old group than in >60 years old group (Figure S2E). Therefore, the SARS-CoV-2 S-specific T cell response is lower and less polyfunctional in aged people after the vaccination with BNT162b2 mRNA vaccine.

Lower thymic function and altered T cell homeostasis found in aged people are associated to a lower T-cell response to the SARS-CoV-2 vaccine

Once we demonstrated that aged people displayed a lower SARS-CoV-2 specific T cell response after vaccination, we investigated the immune defects that might be involved in the diminished response of this vulnerable population. In our group, we previously reported that thymic function failure and inflammation levels independently predict all-cause mortality in healthy elderly people (25). Thus, we studied if these factors could be associated to a lower SARS-CoV-2 vaccine response in aged subjects. Thymic output can be measured through the presence of T cell receptor rearrangement excision circles (TREC) in naïve T cells, indicators of recent thymic emigrants in humans (26). Thus, to determine the thymic function, we have adapted sjTREC measurement with droplet digital PCR (ddPCR). Our results showed a considerably lower level of thymic function in >60 years old people in comparison with <60 years old participants and accordingly, there was a strong association between thymic function and age (Figure 3A). In this line, we observed a correlation between thymic function with naïve CD4+ and CD8+ T cells, and with the naïve CD4+/CD8+ ratio (Figure 3B). In addition, decreased thymic function has been related to the phenomenon called memory inflation, which consists in the alteration of the naïve and memory T cell proportions in the periphery skewing toward memory T cells (27). This phenomenon was reflected in
CD4+ and CD8+ T cell subsets distribution in aged people with >60 years of age, which displayed lower percentages of naïve T cells and higher percentages of memory T cells than young participants (Figure 3C). Three weeks after the vaccination with the first dose, young subjects with <60 years of age showed a trend to decrease the percentages of naïve T cells and to increase memory T cells and a restoration of T cell subset distribution occurred two months after the second dose (Figure 3C). Thus, the differences in naïve and memory T cells between aged and young people observed prior to vaccination were lost after the first dose but were restored two months after the second dose (Figure 3C).

According to lower thymic function and high memory inflation, elevated levels of T cell homeostatic proliferation and activation were found in the elderly people. Specifically, we observed that prior to vaccination, people with >60 years old displayed a higher percentage of activated (HLA-DR+) and proliferating (Ki67+) memory and CM CD4+ T cells comparing with the ones with <60 years old (Figure 3D and 3E). After vaccination with two doses, an increase of HLA-DR+ and Ki67+ CD4+ T cells was observed in young subjects, while older people showed a lack of further activation and proliferation through vaccination (Figure 3D and 3E). Similar results were found in the rest of CD4+ T cell subsets (Figure S3A and S3B) and in some of the CD8+ T cell subsets (data not shown). In this line, thymic function was negatively correlated with T cell activation in most of the subsets and proliferation in naïve CD8+ T cells (Figure S3C). Importantly, thymic dysfunction and the related defects in the homeostasis of T cell compartment found in aged people prior to vaccination, expressed as higher T cell activation and proliferation, along with memory inflation, were correlated with a lower SARS-CoV-2-specific CD4+ and CD8+ T cell response after the two-dose vaccination (Figure 3F-G).
Furthermore, the percentage of activated (HLA-DR+) memory CD4+ T cells was also inversely associated to anti-RBD IgG levels after the first dose and the same trend was observed regarding proliferating (Ki67+) T cells (Figure 3H). Moreover, the vaccination altered the expression of immune checkpoints as LAG-3, PD-1 and TIGIT on CD4+ and CD8+ T cells being higher in <60 years old group compared with the >60 years old one after the first or second dose in most of the T cell subsets (Figure S3D, S3E and S3F). The same result was observed regarding LAG-3 expression within SARS-CoV-2-specific CD4+ T cells, being higher in young subjects after the first dose (Figure 3I). In contrast, PD-1 and TIGIT tended to be lower in the SARS-CoV-2 specific CD4+ T cells from <60 years old group compared to the >60 years old one (Figure 3I). In addition, virus specific T cell response after vaccination was also inversely correlated with the expression of PD-1 in bulk CD4+ T cells before vaccination (Figure S3G).

Other T cells that notably differed between aged and young subjects were CD161+ T cells. These cells mainly produce IL-17 and are known to have an important contribution in pathogen clearance (28). CD161+ T cells presented higher levels in people with <60 years of age comparing with >60 years old ones, independently on the vaccination (Figure 4A). It is remarkable, that the percentage of CD161 expressing CD4+ and CD8+ T cells was positively associated to SARS-CoV-2 S-specific T cell response two months after the vaccination (Figure 4B).

An impaired dendritic cell homing and functional capacity are associated to a lower T-cell response to SARS-CoV-2 vaccine in aged people
In addition to the alteration of the adaptive immunity associated with lower response to the vaccine in the elderly, there is a remodeling of the innate immune system with aging (29). Thus, we next studied plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) (Figure S4A), innate immune cells with a key role in the modulation of T cell response (30). We first observed a decrease in pDC percentages two months after the second dose (Figure S4B). Although we did not find differences between people with >60 and <60 years old in pDC levels (Figure S4B), we observed a considerable difference in pDC functional capacity (Figure 5A). When cells were stimulated in a toll like receptor (TLR)-9-dependent manner by CpG-A, a lower IFN-α production was observed in aged people comparing with young subjects, both after the first and second dose of the SARS-CoV-2 vaccine (Figure 5A, left panel). Interestingly, we also observed how this functional capacity of the pDCs was associated with anti-RBD IgG levels after the first dose (Figure 5A, right panel).

Next, we focused on mDC subsets, including CD1c+, CD16+ and CD141+ mDCs (Figure S4A). Our results showed a higher percentage of CD1c+ mDCs in <60 years old people prior to vaccination and after the first dose (Figure 5B), a mDC subpopulation that modulates CD4+ T cell response (30). In contrast, a notable decrease in CD1c+ mDC percentage was observed only in young people two months after the second dose of the SARS-CoV-2 vaccine (Figure 5B). The same result was found regarding the percentage of integrin-β7 expressing CD1c+ mDCs (Figure S4C, left panel), being integrin-β7 a maker of cell homing to gut. It is remarkable, that the decrease in integrin-β7+ CD1c+ mDCs was correlated with a higher IFN-γ production by TEMRA CD4+ and CD8+ T cells in response to SARS-CoV-2 two months after vaccination (Figure S4C, right panel). Furthermore, higher expression of Indoleamine 2,3-
dioxygenase (IDO) was found on CD1c+ mDCs from young people with <60 years of age in most of the studied time points (Figure 5C) and the percentage of CD1c+ and IDO+ CD1c+ mDCs prior to vaccination were correlated with SARS-CoV-2-specific T cell response two months after the second dose (Figure 5D). Interestingly, although no significant correlation was found, a trend was observed between IDO expression on CD1c and CD16+ mDCs and proliferating (Ki67+) memory CD4+ T cells (Figure S4D). The expression of CD86, PDL1 and CD4 on CD1c+ mDCs, markers related to the modulation of T cell response, was also higher in young people (Figure 5E-G).

It is also known that CD141+ mDCs are involved in the regulation of CD8+ T cell response (30). In this study, higher CD141+ mDC levels were found in the <60 years old group after the first dose, comparing with >60 years old one (Figure S4D). As occurred with CD1c+ mDCs, young people also displayed a higher expression of the co-stimulatory molecule CD86 (Figure 5H, left panel) after vaccination in this subset and this was correlated with SARS-CoV-2 specific cytotoxic response (PRF+) by TEMRA T cells (Figure 5H, right panel). In order to study the functional capacity of mDCs, cells were stimulated with Poly I:C, an agonist of TLR-3. In all subjects, the percentages of activated CD141+ mDCs (CD86+CD40+ and CD83+) were increased following TLR-3-stimulation, before vaccination and after the first dose (Figure S4E). Nevertheless, two months after the second dose, CD141+ mDCs were not successfully stimulated via TLR-3, since they were already activated by the vaccination (Figure S4E). This effect was also observed in the rest of mDC subsets (Figure S4F and G). Even though no differences were found between aged and young people in CD141+ mDC response, a higher functional capacity of CD141+ mDCs prior to vaccination was positively associated with SARS-CoV-2 specific T cell response after vaccination (Figure 5I).
Additionally, other DC markers were altered after the vaccination on different subsets. CCR7, a chemokine receptor that orchestrates DC migration to draining lymph nodes (31), was highly expressed in aged people with >60 years old before vaccination and its expression was increased after the first and second dose mostly in young people, with no response in elderly people (Figure 5J, left and middle panels). A higher CCR7 expression on DCs prior to vaccination was associated to a lower SARS-CoV-2 specific T cell response (Figure 5J, right panel).

Due to their role in the modulation of inflammatory responses, CD16+ mDCs were also studied (32). In general, people with >60 years old displayed higher percentages of CD16+ mDCs than subjects with <60 years old (Figure 6A, left panel). Focusing on the effect of SARS-CoV-2 vaccination, an increase in CD16+ mDC percentages were observed mainly after the first dose but also after the second dose only in aged subjects (Figure 6A). The percentage of CD16+ mDCs and the percentages of CD16+ mDCs expressing integrin β7, IDO and CCR7 were inversely associated with S-specific T cell response (Figure 6B). Hence, our results indicate that the higher levels of CD16+ mDCs, which have a pro-inflammatory function, along with the impaired DC homing and functional capacity found in aged people were associated to a lower T cell response to SARS-CoV-2 vaccine.

Higher monocyte-mediated proinflammatory profile found in aged people is associated to a lower T-cell response to SARS-CoV-2 vaccine

As one of the main players in inflammatory responses and the inflammaging phenomenon (29, 33), monocytes were analyzed in this study. Specifically, previously
studied activation and homing markers (34, 35) were analyzed in classical (CD14++ CD16), intermediate (CD14++ CD16+) and non-classical monocytes (CD14+ CD16++) (Figure S5A). Our results showed that in all subjects, SARS-CoV-2 vaccination induced an increase in the expression of activation markers and toll-like receptors (TLR) in classical and intermediate monocytes, including CD40, TLR-4 (Figure 7A, left panel and Figure S5B), TLR-2 and CD49d (Figure S5C and S5D). Nevertheless, the monocyte activation levels after the first dose were higher in people with <60 years of age than people with >60 years of age (Figure 7A, left panel and Figure S5B). It is remarkable, that the expression of CD40 and TLR-4 before vaccination were positively correlated to SARS-CoV-2-specific T cell response two months after the second dose (Figure 7A, right panel). The expression of CCR5, a monocyte chemokine receptor, is modulated after activation (36). Our results showed that CCR5 expression before vaccination was higher in monocytes from young than in aged people (Figure 7B, left panel). However, it was downregulated after the first dose of SARS-CoV-2 vaccination, being this decrease less pronounced in aged people (Figure 7B, right panel). Importantly, basal CCR5 expression was also directly correlated to IFN-γ production by CM CD4+ and TEMRA CD8+ T cells after SARS-CoV-2 vaccination (Figure 7C). Regarding other monocyte chemokine receptors, two-dose vaccination induced a decrease in the percentage of intermediate monocytes expressing CCR2 and CD11b and non-classical monocytes expressing CX3CR1, being these percentages lower in <60 years old people than in >60 years old ones (Figure 7C and D and Figure S5E). The lower percentages of monocytes expressing these chemokine receptors two months after the second dose of vaccination was inversely correlated with SARS-CoV-2 T cell response and regarding CD11b, also with anti-RBD IgG levels (Figure E-G). Furthermore, the expression of
CX3CR1 in non-classical monocytes prior to vaccination was also inversely associated to the virus-specific T cell response (Figure 7F, right panel). Moreover, monocyte tissue factor (CD142) expression is known to be induced by several inflammatory stimuli (37, 38). Here, we found an increase in the expression of tissue factor on classical monocytes after SARS-CoV-2 vaccination in both groups (Figure S5F, left panel). In addition, tissue factor expression levels were higher on intermediate monocytes from young subjects after the first dose (Figure S5F, right panel).

It has been previously reported that intermediate monocytes are expanded in the blood of patients with systemic infections (39). Here, we found a considerable increase in the percentage of intermediate monocytes, along with a decrease in classical monocytes, after the two-dose SARS-CoV-2 vaccination only in people with >60 years old (Figure 8A). No differences were found prior vaccination or after the first dose (Figure S5G). Intermediate monocytes are known to secrete TNF-α, IL-1β, IL-6, and CCL3 upon TLR stimulation (39). In this study, we stimulated cells in a TLR-4 dependent manner by adding lipopolysaccharide (LPS). Importantly, vaccinated aged people showed a higher production of IL-6, IL-1α and TNF-α by monocytes upon LPS stimulation comparing with young subjects, mainly after the second dose (Figure 8B).

Lastly, we discovered that participants which did not show a cytotoxic SARS-CoV-2 specific T cell response (PRF-) produced higher levels of inflammatory cytokines by monocytes after vaccination, than the ones presenting cytotoxic T cells (PRF+) after SARS-CoV-2 vaccination (Figure 8C). In summary, although monocytes from aged people showed lower levels of activation and homing after the vaccination, produced higher levels of proinflammatory cytokines upon LPS stimulation which was inversely associated to SARS-CoV-2 specific T cell response.
Aging is associated with impaired COVID-19 vaccine response (20). We confirmed and extended that the age-related immunological defects were characterized mostly by a lower magnitude and polyfunctionality of SARS-CoV-2 specific T cell response. The adaptive and innate immune factors behind these defects in aged people included an alteration in T cell homeostasis parameters fueled by lower thymic function and higher T cell activation and proliferation, dendritic cell dysfunction and a higher proinflammatory profile in circulating monocytes.

In spite of the efficacy of BNT162b2 mRNA vaccine to prevent severe COVID-19 outcomes, vulnerable populations as elderly people remain at risk. Several studies have reported lower humoral response in aged subjects following vaccination (19–21) (22, 23). In accordance to these findings, we observed an inverse association between anti-RBD IgG levels with age two months after vaccination. Moreover, we also found a diminished specific T cell response to SARS-CoV-2 vaccine in aged people, confirming previous results (20, 21). In addition to the magnitude, high quality of SARS-CoV-2 specific T cell function is required to achieve an effective vaccine response, which has not been completely determined yet. We found that specific T cells in addition to produce different cytokines, also exhibited a cytotoxic response to the vaccination, which is diminished in elderly. Although young people showed a higher polyfunctional response than aged ones, BNT162b2 mRNA vaccine did not induce a high polyfunctional T cell immunity in general. This could be one of the key factors that might explain the absence of vaccine efficacy to avoid new infections along time, independently of the virus variant of concern (VOC).
Age-related changes in the immune system, known as immunosenescence, cause a subclinical immune deficiency that involves a reduced antiviral function and vaccine response (5). One of the most known changes of the immune aging is the involution of the thymus (40) (27). In our study, aged people exhibited thymic dysfunction and the subsequent memory inflation, reproducing previous findings (41). Remarkably, it has been described that thymic function failure predicts all-cause mortality in healthy aged people (25) and has a relevant role in viral infections such as HIV-1 infection (26, 42, 43). Moreover, it has been suggested that thymic aging might have an important implication in COVID-19 disease severity (44, 45). Importantly, we discovered that the thymic dysfunction, along with the memory inflation and a higher homeostatic T cell proliferation and activation found in aged people, were correlated to a lower response to SARS-CoV-2 vaccination. Accordingly, a lower activity of the thymus has been previously associated to diminished responsiveness to vaccination against other viruses as yellow fever virus (46). In addition, thymic dysfunction was associated to a higher homeostatic T cell proliferation and activation. In fact, the age-dependent shift in the T cell population from naïve to memory phenotype induces homeostatic T cell proliferation to compensate the diminished T cell thymic output (47, 48). The higher T cell activation status found in aged people prior vaccination might be the reason why there is a lack of further activation of T cells after SARS-CoV-2 vaccination in this population. This was reflected in a lower increase in the expression of T cell activation markers as HLA-DR in aged people after vaccination, but also lower expression of checkpoint receptors as LAG-3 in both bulk and SARS-CoV-2 specific CD4+ T cells, a marker that is overexpressed after cellular activation (49).
One of the most remarkable findings of our study is the tight association of CD161 expression levels with age. CD161 is a C-type lectin receptor which is expressed in both T and NK cells (50). CD161+ T cells has been associated with IL-17 production and hence with pathogen clearance (51). In fact, we have previously observed that CD161+ CD8+ T cells were related with HIV and hepatitis C virus (HCV)-specific T cells polyfunctionality, which is essential for HIV spontaneous control and HCV spontaneous clearance (28). Results presented herein suggest the absence of CD161 marker as a hallmark of T cell immunosenescence in accordance with recent findings, which show an inverse correlation of CD161 expression on CD8+ T cells with age independently of CMV infection (52). Importantly, a reduction in the frequency of CD161+ CD8+ T cells was found in peripheral blood of severe COVID-19 patients (53). In this study, we found CD161 expression on T cells was tightly associated with SARS-CoV-2 vaccine response, highlighting this molecule as a potential target for immunotherapeutic strategies for age-related disease therapies and vaccine response in the elderly.

The regulation of the immune response highly depends on the function of DCs (30); however, their role in immune aging needs to be better understood. In this work, we found an altered DC subset distribution and an impaired DC homing and function with aging. The levels of CD1c+ mDCs, cells responsible for the modulation of CD4+ T cell response, were lower in aged people prior to vaccination and this was related to a lower SARS-CoV-2 T cell response after vaccination. Accordingly, Schulz et al. previously observed an association between DC numbers and the response to yellow fever vaccine (46). Furthermore, the lower numbers of CD1c+ mDCs and integrin-β7 expressing CD1c+ mDCs observed after the second dose indicates that vaccination may induce a CD1c+ mDC migration to gut and probably to other tissues. In this line, a
decrease in peripheral blood CD1c+ mDC numbers has been found in patients with severe COVID-19, due to a migration of these cells from blood to the lungs (54, 55).

Interestingly, the vaccine-induced CD1c+ homing was barely observed in aged people and this was also associated to a decreased SARS-CoV-2 specific T cell response, suggesting that aging might affect the capacity of CD1c+ mDCs to migrate and consequently also their ability to modulate T cell function. In this line, a lower expression of receptors as CD86 in aged people could be indicative of a less efficient CD1c+ mDC activation and T cell co-stimulation after vaccination.

In addition to homing, DC function is also altered with aging. In response to TLR-3, TLR-7/8, and TLR-9 ligand stimulation, CD141+ mDCs and pDCs from aged subjects secreted lower levels of IL-6, IL-12, and TNF-α (56). Other studies have reported, that in response to influenza A virus infection and West Nile infection, pDCs from older adults produced less type I IFN (57). Accordingly, our results showed a lower TLR-9-dependent IFN-α production by pDCs in aged people, both before and after the vaccination against SARS-CoV-2. According to this, patients with COVID-19 presented a lower TLR-9-mediated IFN-α production than healthy donors (54). This might be of great importance, since type I IFNs control innate and adaptive immune system and induce cells’ antiviral state via the upregulation of IFN-stimulated genes that inhibit the replication and spreading of viruses (58). Remarkably, we described that CD141+ mDCs may also have an important implication in SARS-CoV-2 vaccination immunity, which is one of the cell types that is known to be depleted in COVID-19 patients and are important for disease progression (55, 59). Here, we discovered that TLR-3-mediated CD141+ mDC activation capacity was directly associated to SARS-CoV-2 T cell response.
following vaccination. This is in accordance with the important function of CD141+ mDCs in antigen presentation to T cells (60, 61).

Other important innate immune cells that might have a relevant role in vaccine response are monocytes. In this study, we reported that SARS-CoV-2 vaccination caused monocyte activation and homing, reflected by higher expression of activation markers (CD40, TLR-4, TLR-2 and CD49d) and lower percentage of CCR2, CD11b and CX3CR1 expressing intermediate and non-classical monocytes after vaccination. It has been described, that in addition to DCs, these monocyte subsets also migrate from bloodstream to lungs in patients with COVID-19 (55). However, vaccine-induced monocyte homing was found mainly in young people. As same as we observed in CD1c+ mDCs, less monocyte homing was associated to a lower specific T cell response to vaccination. Although monocytes have not a main role in the modulation of T cell response, monocytes are known to have the ability to prime tissue resident T cells via cytokine production (62). Thus, a deficit in monocyte migration to inflammatory sites might also negatively affect T cell response.

One of the key age-related immune defects is the phenomenon called inflammaging, a persistent increase in basal pro-inflammatory phenotype found in the elderly (4). Monocytes are one of the principal players of inflammaging (29, 33). Inflammation is a critical factor in COVID-19 progression where monocyte-driven cytokine storm induces a hyper-inflammatory phenotype leading to a more severe symptomatology in COVID-19 patients (63). Here, we discovered a higher TLR-4-mediated pro-inflammatory cytokine production by monocytes from aged people after SARS-CoV-2 vaccination and importantly, this cytokine production was inversely correlated to specific T cell
This is in accordance to previously published studies, describing the role of inflammatory monocytes in the suppression of vaccine responses (64, 65). In fact, increased gene expression of inflammatory responses and TNF-α signaling via NFκβ were reported after COVID-19 vaccination (66). Importantly, plasma TNF-α levels in aged people were associated to a poorer antibody response following SARS-CoV-2 vaccination (21). According with our results from monocytes, aged people showed increased numbers of CD16+ mDCs, a DC subset that also participate in inflammatory responses (32). This higher CD16+ mDC levels were inversely associated to SARS-CoV-2 specific T cell response after vaccination. Altogether, a higher capacity of monocytes to produce pro-inflammatory cytokines, increased CD16+ mDC numbers and even the higher T cell activation status found in aged people are probably associated to the inflamming phenomenon, which at the same time is related to lower vaccine immunity.

In the present study, numerous age-related immune deficits associated to a lower SARS-CoV-2 vaccination response were described. It may be interesting to carry out the same determinations long-term after SARS-CoV-2 vaccination, to study how these immune alterations might be contributing to a less durable protection after COVID-19 vaccination found in aged people. Nevertheless, the described age-associated immune defects are already observed two months after the second dose, therefore, these defects are expected to be maintained or even increase with a longer follow up and might explain why waning in mRNA vaccine effectiveness occurs at a greater rate among older people. Another limitation of the study might be the sample size. However, we performed an exhaustive and comprehensive analysis of age-associated immune deficits and the differences and associations among the contrasts were very
clear. Moreover, functional assays were performed with PBMCs, instead of isolated
cells, and therefore, some of the alterations found in the studied cells responses might
be influenced by bystander cells. However, the analysis of functional capacity using
PBMCs reflects better what is occurring in physiological conditions. Lastly, another
limitation of this work could be that we only studied the response to BNT162b2 mRNA
vaccine. Nevertheless, it is one of the most administered COVID-19 vaccines and it
would not be surprising that other vaccines present similar results, especially other
mRNA vaccines.

In conclusion, we describe age-related innate and adaptive immune deficits associated
to a lower SARS-CoV-2 vaccination response. Based on all the results from the study,
we suggest that the putative causal drivers of the lower vaccine response in aged
people are: 1) thymic dysfunction, which induces a memory inflation (less naïve T cells
and higher memory T cells) and directly influence T cell response to the vaccine in aged
people; 2) defective DC migration and activation, that cause a lower DC-mediated T
cell co-stimulation and therefore a lower T cell response to the vaccine; 3) the
inflammaging, induced by a higher production of pro-inflammatory cytokines by
monocytes and accompanied by a higher activation of the immune system, which
causes an inefficient further activation of the immune cells in response to new
antigens or in this case to the vaccine. These findings contribute to a better
understanding of why aged people are less capable to respond to SARS-CoV-2
vaccination and might be relevant for the improvement of the current vaccination
strategies, especially in this vulnerable population, and for the development of more
efficient prototypes for the general population.
MATERIALS AND METHODS

STUDY PARTICIPANTS

Fifty-four participants vaccinated with BNT162b2 mRNA vaccine against SARS-CoV-2 were included in this study. Participants were stratified by age: <60 categorized as young (n=33) and ≥60 categorized as aged (n=21), the median ages of young and aged subjects were 29 [IQR, 26-49] and 73 [72-74], respectively. Young participants were workers from the Institute of Biomedicine of Seville and aged participants were community volunteers from Seville, Dos Hermanas (Seville, Spain) and Rota (Cadiz, Spain). Inclusion criteria included subjects with self-sufficient health status and participants were excluded if they had a diagnose of dementia, active infections or hospital admission during the last six months. Three young subjects were excluded from the study due to a positive result for SARS-CoV-2 PCR or SARS-CoV-2 RBD-specific antibodies prior to vaccination. Peripheral blood samples were extracted from February to November 2021.

CELL AND PLASMA ISOLATION

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from study subjects’ blood. PBMCs were isolated using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (with Sodium Heparin, BD Biosciences) in a density gradient centrifugation at the same day of blood collection. CPTs were centrifuged at 3000 rpm for 20 min at room temperature. Afterwards, PBMCs were cryopreserved in freezing
medium (90% of fetal bovine serum (FBS) (Gibco) + 10% dimethyl sulfoxide (DMSO) (PanReac AppliChem) in liquid nitrogen until further use. Plasma samples were obtained using BD Vacutainer™ PET EDTA Tubes centrifugation at 3000 rpm for 5 min, aliquoted and cryopreserved at -80°C until further use.

**CELL STIMULATION**

PBMCs were thawed, washed using RPMI 1640 (Gibco) and rested for 1 h in 0.25 µL/mL DNase I (Roche Diagnostics) containing R-10 complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin G, 100 l/ml streptomycin sulfate (Gibco), and 1.7 mM sodium L-glutamine (Lonza)).

**SARS-CoV-2 specific T cell response**

To analyze the specific T cell response to SARS-CoV-2, 1.5 × 10^6 PBMCs were *in vitro* stimulated for 6 h at 37°C in R-10 medium with overlapping peptides of protein Spike (PepMix™ SARS-CoV-2; Spike Glycoprotein, from JPT). 1.5 × 10^6 PBMCs incubated with the proportional amount of DMSO were included in each experiment as a negative control. The stimulation was performed in the presence of 10 µg/mL of brefeldin A (Sigma Chemical Co) and 0.7 µg/mL of monensin (Golgi Stop, BD Biosciences) protein transport inhibitors, anti-CD107a-BV650 (clone H4A3; BD Biosciences) monoclonal antibody and purified CD28 (clone CD28.2) and CD49d (clone 9F10) (BD Biosciences) as previously described (67, 68). Intracellular cytokines and cytotoxicity markers were analyzed by multiparametric flow cytometry. Specific T cell response was defined as the frequency of cells expressing intracellular cytokines and/or cytotoxicity markers.
after the stimulation with Spike peptides minus the levels of this response in the unstimulated condition (background subtraction).

**Monocyte stimulation**

1 × 10^6 PBMCs were *in vitro* stimulated in a 48-well plate for 5 h at 37°C with 0.5 µL/ml of lipopolysaccharide (LPS, Invivogen) in R-10 medium, including 1 × 10^6 PBMCs without any stimulation as a negative control. 0.7 µg/mL of monensin (Golgi Stop, BD Biosciences) was added to all experimental conditions. Intracellular cytokines were analyzed by flow cytometry.

**Myeloid Dendritic Cell stimulation**

0.5 × 10^6 PBMCs were *in vitro* stimulated in a 24-well plate for 24 h at 37°C with 2 µL/ml of polyinosinic:polycytidylic acid (Poly-I:C, InvivoGen) in R-10 medium. 0.5 × 10^6 PBMCs incubated without stimulus were included as a negative control. Surface expression of activation markers were analyzed by flow cytometry.

**pDC stimulation culture and quantification of IFN-α production**

0.5 x 10^6 thawed PBMCs were incubated at 37°C and 5% CO_2 during 18 hours in R-10 medium with or without 1 µM of the TLR9 agonist CpG-A (InvivoGen). After incubation, cells were pelleted and the supernatants conserved at -80°C for the subsequent quantification of interferon (IFN)-α production by ELISA according manufacturer’s instructions (PBL Interferon Source).

**MULTIPARAMETRIC FLOW CYTOMETRY**
In general, for \textit{ex vivo} phenotyping and functional assays, PBMCs were washed (1800 rpm, 5 min, room temperature) with Phosphate-buffered saline (PBS (Gibco)). PBMCs were then incubated 35 min at room temperature (RT) with a viability marker (LIVE/DEAD Fixable Aqua or Violet Dead Cell Stain; Life Technologies) and all the extracellular antibodies (see below). PBMCs were washed and fixed and permeabilized with BD Cytofix/CytoPerm (BD Biosciences) at 4°C for 20 min or Fixation/Permeabilization Buffer Set (eBioscience) at 4°C for 45 min following the manufacturer’s protocol. Then, cells were stained at 4°C for 30 min with intracellular antibodies (see below) and washed. Finally, cells were fixed for 20 minutes at 4°C with 4 % paraformaldehyde solution (PFA (Sigma-Adrich)).

To assay T cell specific response, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-DUMP-channel-BV510 (CD14-clone MφP9, CD19 clone SJ25C1, CD56 clone NMCAM16.2), anti-CD8-APC (clone SK-1), anti-CD3-BV711 (clone SP34-2), anti-CD45RA-FITC (clone L48), anti-CD27-APCH7 (clone M-T271) anti-PD-1-BV786 (CD279, clone EH12-1) (BD Bioscience) and anti-TIGIT-PerCPCy5.5 (clone A15153G) and anti-LAG3-BV605 (clone 11C3C65) (BioLegend). They were permeabilized and fixed with Cytofix/CytoPerm buffer (BD Bioscience). Cells were intracellularly stained with: anti-IL-2-BV421 (clone MQ1-17H12), anti-IFN-γ-PE-Cy7 (clone B27) (BD Bioscience), anti-TNF-α-AF700 (clone Mab11) (BD Pharmingen) and anti-Perforin-PE (clone B-D48) (BioLegend). For T cell phenotyping, cells were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti CD8-PerCP-Cy5.5 (clone SK1), anti-CD45RA-PeCy7 (clone L48), anti-CD3-BV711 (clone SP34-2) (BD Bioscience), anti-HLA-DR-BV570 (clone L243), anti-CD161-BV421 (clone HP-3G10) (BioLegend); permeabilized and fixed with
Fixation/Permeabilization buffer (eBioscience™); and intracellularly stained with: anti-Ki67 FITC (clone 11F6) (BioLegend). T cells were gated based on the CD3 and CD8 expression. Each subset (Total Memory, Memory; Central Memory, CM; Effector Memory, EM; and terminally differentiated effector memory, TEMRA) was gated based on CD45RA and CD27 expression (Figure S2A).

To assay monocytes functionality, PBMCs were extracellularly stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies), anti-DUMP-channel-V450 (CD3 clone SP34-2, CD19 clone HIB19, CD20 clone L27, CD56 clone B159), anti-CD14-BV650 (clone M5E2), anti-CD16-PeCF594 (clone 3G8), anti-HLA-DR-BV570 (clone L243), (BioLegend); permeabilized and fixed with BD Cytofix/CytoPerm (BD Biosciences); and intracellularly stained with anti-IL-6-Pe (clone MQ2-6A3), anti-IL-1α-FITC (clone AS5), anti-TNF-α-AF700 (clone MAb11) (BD Biosciences). To assay monocytes phenotyping ex vivo, PBMCs were extracellularly stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies), anti-DUMP-channel-V450 (CD3 clone SP34-2, CD19 clone HIB19, CD20 clone L27, CD56 clone B159), anti-CD14-BV650 (clone M5E2), anti-CD16-PeCF594 (clone 3G8), anti-CD142-Pe (clone HTF), anti-CCR5-APC-Cy7 (clone 2D7/CCR5) (BD biosciences), anti-HLA-DR-BV570 (clone L243), anti-TLR2-FITC (clone TL2.1), anti-CD40-APC (clone HB14), anti-CX3CR1-PerCPCy5,5 (clone 2A9-1), anti-CCR2-BV605 (clone K036C2), anti-CD49d-BV711 (clone 9F10) (BioLegend), and anti-CD11b-AF700 (clone VIM12) (Life Technologies, Thermo Fisher Scientific). Monocytes were gated based on the CD14 and HLA-DR markers and non-classical, intermediate and classical subsets were gated based on CD14 and CD16 expression (Figure S5A).
To assay mDC functionality, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-CD11c-BV650 (clone B-ly6), anti-HLA-DR-BV570 (clone L243), anti-Lin-2-FITC (CD3 clone SK7, CD19 clone SJ25C1, CD20 clone L27, CD14 clone MφP9 and CD56 clone NCAM16.2), anti-CD16-BV605 (clone 3G8) (BD Biosciences), anti-CD1c-APCCy7 (clone L161), anti-CD141-PeCy7 (clone M80), anti-CD86-BV421 (clone 2331 (FUN-1)), anti-CD40-APC (clone HB14) (BioLegend) and anti-CD83-AF700 (clone HB15) (Invitrogen); permeabilized and fixed with BD Cytofix/CytoPerm (BD Biosciences); and intracellularly stained with anti-IDO-Pe (clone eyedio) (eBioscience). For ex vivo DC phenotyping, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-CD11c-BV650 (clone B-ly6), anti-HLA-DR-BV711 (clone G46-6), anti-Lin-2-FITC, anti-CD16-BV605 (clone 3G8), anti-CCR7-BV786 (CD197) (clone 3D12), anti-CD86-BV421 (clone 2331 (FUN-1)), anti-PD-L1 PeCF594 (CD274) (clone MIH1), anti-Integrin-β7-APC (clone FIB504) (BD Biosciences), anti-CD4-PerCPCy5,5 (clone OKT4), anti-CD1c-APCCy7 (clone L161), anti-CD141-PeCy7 (clone M80) (BioLegend) and anti-CD123-AF700 (clone 32703) (R&D Systems) antibodies. Cells were permeabilized and fixed with Fixation/Permeabilization buffer (eBioscience™); and intracellularly stained with anti-IDO-Pe (clone eyedio) (eBioscience). DCs were identified by the expression of HLA-DR and the lack of expression of Lin-2. pDCs and mDCs were gated based on the CD123 and CD11c expression, respectively. mDCs subsets were gated using according to CD16, CD1c and CD141 expression (Figure S4A).

Multiparametric flow cytometry were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences), acquiring 0.5-1 × 10^6 events. Data were analyzed using the FlowJo 10.7.1 software (Treestar, Ashland, OR).
QUANTIFICATION OF ANTI-RBD AND ANTI-IFN-α IgG LEVELS

Anti-RBD IgG SARS-CoV-2 levels were measured by recombinant (r)RBD specific ELISA as previously described (Grifoni et al., 2020; Rydzynski Moderbacher et al., 2020; Sette and Crotty, 2020; Amanat et al., 2021). Briefly, Nunc Maxisorp flat-bottomed 96-well plates (ThermoFisher Scientific) were coated with 1μg/mL of rRBD protein of the spike (S) antigen of SARS-CoV-2 (Sino Biological, #40592-V08H), overnight at 4°C. The following day, plates were blocked with 3% milk in PBS containing 0.05% Tween-20 for 120 min at RT. Plasma samples were heat inactivated at 56°C for 20 min complement activity. Human plasma samples were diluted at 1:50, 1:100, 1:200, 1:400, or 1:800 in 1% milk containing 0.05% Tween-20 in PBS and incubated for 90 min at room temperature. Plates were washed four times with 0.05% PBS-Tween-20. Human serum standard reference material of anti-SARS-CoV-2 immunoglobulin (first WHO International Standard and International Reference Panel for anti-SARS-CoV-2 immunoglobulin from NIBSC, UK, NIBSC code: 20/150) was used as standard curve to titer anti-SARS-CoV-2 IgG antibody in plasma samples. Human serum standard was added to the plates and serially diluted (twofold dilutions) in 1% milk containing 0.05% Tween-20 in PBS. Pooled plasma samples (NIBSC, UK, NIBSC code: 20/142) obtained from healthy blood donors before 2019 was used as negative control plasma. Secondary antibodies, streptavidin-horseradish peroxidase-conjugated mouse anti-human IgG (Hybridoma Reagent Laboratory, Baltimore, MD,) was used at a 1:5,000 dilution in 1% milk containing 0.05% Tween-20 in PBS. Plates were washed four times with 0.05% PBS-Tween-20. The plates were developed using fast o-Phenylenediamine.
dihydrochloride Peroxidase Substrate (Sigma-Aldrich), the reaction was stopped using 3 M HCl, and the optical density at 490 nm (OD490) was read on a Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific) within two hours. Anti-SARS-CoV-2 IgG antibody titers for each donor were calculated as Binding Antibody Units (BAU)/ml according to the manufacturers’ information regarding the WHO Standard and was determined based on sigmoidal dose-response nonlinear regression, 4PL, using GraphPad Prism, version 8.0 (GraphPad Software, Inc.). For the measurement of anti-IFN-α IgGs, another ELISA assay was performed as previously described (69). In this case, plates were coated with 2ug/mL of IFN-α2a (Miltenyi Biotec) and plasma samples were diluted 1:10. Eight non-vaccinated healthy donors and three severe COVID-19 patients from a previous study of the group (68) were used as negative and positive controls respectively.

DNA EXTRACTION AND TREC MEASUREMENT BY ddPCR

The extraction of the genomic DNA from frozen PBMCs was performed using a blood DNA minikit (Omega; Bio-Tek). The DNA concentration was determined by Qubit assay according to the manufacturer’s protocol (ThermoFisher Scientific).

T cell receptor rearrangement excision circles (TRECs) were quantified from extracted DNA by droplet digital PCR (ddPCR) (Bio-Rad) based on a previously modified method (70). 20 µM of primer for signal joint (sj)-TREC (DTF7, 5’→3’: AGGCTCTGTCTAGTGATAAC; DTR66, 5’→3’: TGACATGGAGGGCTGAAC), 10 µM of probe (PB1, 5’→3’: 6FAM-TGGGAGTTGGACGCCAGAGG-BHQ1; SD1, 5’→3’: HEX-CACCCCTCTGTCCCCACA- BHQ1) and ddPCR Supermix for probes no dUTP (Bio-Rad) were used. The reference gene used was RPP30 (two copies per cell) (Forward, 5’→3’:
GATTGGACCTGCGAGCG; Reverse, 5′→3′: GCGGCTGTCTCC ACAAGT; Probe, 5′→3′: VIC-CTGACCTGAAGGCTCT-BHQ1). The ddPCR conditions were: 10 min at 95 °C, 40 cycles of 30 sec at 94 °C, 1 min at 59 °C and 10 min at 98°C. Bio-Rad QuantaSoft software v.1.7.4 was used for determining the TREC/10^6 cells.

**STATISTICS**

Non-parametric statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 25.0; SPSS, Inc.), RStudio Version 1.3.959 and GraphPad Prism version 8.0 (GraphPad Software, Inc.). Polyfunctionality pie charts were constructed using Pestle version 1.6.2 and Spice version 6.0 (71). Median and interquartile ranges were used to describe continuous variables and percentages to describe categorical variables. ROUT method was utilized to identify and discard outliers. Differences between aged and young groups were analyzed by two-tailed non-parametric Mann-Whitney U test. The non-parametric Wilcoxon test was used to analyze differences between time points. The Spearman test was used to analyze correlations between variables. For multiple comparisons, Friedman test was applied, including Bonferroni correction. Permutation test was used to assess differences between pie charts using Spice software. Hmisc and corrplot packages were used in R by Spearman method to calculate correlations between pairs of variables and plot the correlation matrix figures. Lateral intensity bar from red to blue, next to correlation matrixes, represents (P, p) rho coefficient value of the Spearman test. All differences with a P value < 0.05 were considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
The study was approved by the Ethics Committee of the Virgen del Rocio University Hospital (protocol code “COVIMARATON”; internal code 0896-N-20). Written informed consent was received prior to participation.

**AUTHOR CONTRIBUTIONS**


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Figure 1. Association of SARS-CoV-2 specific IgG levels with age

(A) Anti-RBD IgG levels (Binding Antibody Units (BAU)/mL) in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D). (B) Correlation of anti-RBD IgG levels with age in all the study participants after the first dose (left) and after the second dose (right). Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 54). Friedman test was applied in panel A (>60 years old, p= <0.0001; <60 years old, p= <0.0001).
Figure 2. Aged people show a lower and less polyfunctional SARS-CoV-2 spike-specific CD4+ and CD8+ T cell response after the vaccination

(A and B) Bar graphs presenting the percentage of IFN-γ+, CD107a+ and PRF+ cells within memory, CM, EM and TEMRA CD4+ (A) and CD8+ (B) T cells after S-specific SARS-CoV-2 stimulation, comparing >60 years old (red) and <60 years old (blue) subjects three weeks after the first dose (1D) and two months after the second dose (2D) of SARS-CoV-2 vaccine (right). Pseudocolour dot plot graphs show a representative data of memory CD4+ T cells from an >60 years old and <60 years old donor two months after vaccination (left). (C) Pie charts representing SARS-CoV-2 S-specific memory CD4+ T cell polyfunctionality. Each sector represents the proportion of S-specific CD4+ T cells producing two (green) or one (blue) functions. Arcs represent the type of function (CD107a, IFN-γ, IL-2, PRF and TNF-α) expressed in each sector. (D) Bar graphs showing the percentage of EM and CM CD4+ T cells expressing different combinations of studied functions (CD107a, IFN-γ, IL-2, PRF and TNF-α) comparing >60 years old (red) and <60 years old (blue) subjects after the first (1D) and the second (2D) dose. Mann-Whitney U, Wilcoxon and Permutation tests were used (n= 41).
Figure. 3. Lower thymic function and altered T cell homeostasis found in aged people are associated to a lower T cell response to the SARS-CoV-2 vaccine

(A and B) Bar graphs showing TREC/10^6 cells as a measure of thymic function in >60 years old and <60 years old participants prior to vaccination (A, left) and the correlation of the TREC/10^6 cells with age (A, right), naïve CD4+ T cells (B, left), naïve CD8+ T cells (B, middle) and naïve CD4+/CD8+ T cell ratio (B, right). (C) Bar graphs representing the percentage of naïve and memory CD4+ and CD8+ T cells in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D). (D and E) Bar graphs representing the percentage of memory and CM CD4+ T cells expressing HLA-DR (D) and Ki67 (E) >60 years old (red) and <60 years old (blue) participants at the three time points (right). Pseudocolour dot plot graphs show representative data of memory CD4+ T cells from an >60 years old (red) and <60 years old (blue) donor expressing HLA-DR (D) and Ki67 (E) before vaccination (left). (F and G) Correlation matrixes representing associations of SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing IFN-γ or cytotoxicity markers two months after the second dose of vaccination with TREC/10^6 cells and naïve T cells (F) and with the percentage of HLA-DR+ and Ki67+ CD4+ T cells (G) before vaccination in all participants. (H) Correlation plots of anti-RBD IgG levels after the first dose of vaccination with the percentage of HLA-DR+ and Ki67+ CD4+ T cells before vaccination. (I) Bar graphs representing the percentage of SARS-CoV-2-specific CD4+ T cells expressing LAG-3 in >60 years old (red) and <60 years old (blue) participants at the three follow up time points. Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 32). Friedman test was applied in panels C (Naïve CD4+ T cells: >60 years old, p= 0.06; <60 years old, p=0.074; Memory CD4+ T cells: >60 years old, p= 0.071; <60 years old, p=0.074; Naïve CD8+ T cells: >60 years old, p= 0.307; <60 years old, p= 0.015; Memory CD8+ T cells: >60 years old, p= 0.035; <60 years old, p= 0.091), D (Memory CD4+ T cells: >60 years old, p= 0.441; <60 years old, p= 0.022 and CM CD4+ T cells: >60 years old, p= 0.529; <60 years old, p= 0.022) and E (Memory CD4+ T cells: >60 years old, p= 0.273; <60 years old, p= 0.074 and CM CD4+ T cells: >60 years old, p= 0.657; <60 years old, p= 0.091).
**Figure 4.** CD161 expressing T cell levels are associated to a higher SARS-CoV-2 vaccine T cell response

(A) Bar graphs representing the percentage of memory and CM CD4+ T cells expressing CD161 in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) (right). Pseudocolor dot plot graphs show representative data of memory CD4+ T cells from a >60 years old and <60 years old donor expressing CD161 before vaccination (left). (B) Correlation matrix representing associations of the percentage of CD161+ T cells before vaccination with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing IFN-γ or cytotoxicity markers two months after the second dose of vaccination in all participants. Mann-Whitney U, Wilcoxon and Spearman tests were used (n=32). Friedman test was applied in panel A, right (Memory CD4+ T cells: >60 years old, p= 0.091; <60 years old, p= 0.368 and CM CD4+ T cells: >60 years old, p= 0.159; <60 years old, p= 0.549).
Figure 5

**A**

>60 years old

<60 years old

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**B**

>60 years old

<60 years old

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**C**

>60 years old

<60 years old

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**D**

>60 years old

<60 years old

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**E**

>60 years old

<60 years old

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**F**

>60 years old

<60 years old

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**G**

>60 years old

<60 years old

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**H**

>60 years old

<60 years old

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**I**

>60 years old

<60 years old

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**J**

>60 years old

<60 years old

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Figure 5. An impaired DC homing and functional capacity are associated to a lower T cell response to SARS-CoV-2 vaccine in aged people

(A) Dot plots showing IFN-α production through CpG-A stimulation for 18 hours in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) (left). Correlation analysis of IFN-α production with anti-RBD IgG levels three weeks after the first dose of vaccination (right). (B and C) Bar graphs representing the percentage of CD1c+ and IDO+ CD1c+ mDCs in >60 years old (red) and <60 years old (blue) participants at the three follow up time points. (D) Correlation matrix showing associations between the percentages of CD1c+ mDCs and IDO+ CD1c+ mDCs before vaccination with SARS-CoV-2 S-specific T cells expressing cytokines or cytotoxicity markers two months after the second dose. (E-G) Dot plots showing the percentage of CD1c+ mDCs expressing CD86 (E), PDL-1 (F) and CD4 (G) in >60 years old (red) and <60 years old (blue) participants at the three time points. (H) Dot plots showing the percentage of CD141+ mDCs expressing CD86 (H, left) in >60 years old (red) and <60 years old (blue) participants at the three time points. Correlation plot between the percentage of CD86+ CD141+ mDCs before vaccination and the percentage of S-specific PRF+ TEMRA CD4+ and CD8+ T cells two months after the second dose (H, right). (I) Correlation matrix showing associations between the percentage of CD141+ mDCs expressing activation markers after TLR-3 stimulation for 24 hours with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing cytotoxicity markers. (J) Dot plots showing the percentage of CCR7+ mDCs in >60 years old (red) and <60 years old (blue) participants in the three follow up time points (left and middle panels) and correlation matrix representing associations of the percentage of mDCs expressing CCR7 with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing cytokines or cytotoxicity markers two months after the second dose (right panel). Mann-Whitney U, Wilcoxon and Spearman tests were used (n = 32). Friedman test was applied in panel A (>60 years old, p = 0.801; <60 years old, p = 0.717), B (>60 years old, p = 0.169; <60 years old, p = <0.0001), C (>60 years old, p = 0.147; <60 years old, p = 0.027), E (>60 years old, p = 0.018; <60 years old, p = 0.086), F (>60 years old, p = 0.381; <60 years old, p = 0.013), G (>60 years old, p = 0.042; <60 years old, p = 0.034), H (>60 years old, p = 0.223; <60 years old, p = 0.234) and J, left (CD1c mDCs: >60 years old, p = 0.121; <60 years old, p = 0.001 and CD141 mDCs: >60 years old, p = 0.459; <60 years old, p = 0.001).
Figure 6. Higher CD16+ myeloid dendritic cell percentage from aged people is associated to a lower T cell response to SARS-CoV-2 vaccine

(A) Bar graphs representing the percentage of CD16+ mDCs in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination. (B) Correlation matrix showing associations between the percentage of CD16+ mDCs and CD16+ mDCs expressing integrin-β7 and IDO with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing cytotoxicity markers. Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 32). Friedman test was applied in panel A, left (>60 years old, p= 0.01; <60 years old, p= 0.148).
Figure 7

A. % CD40+ classical (PRE, 1D, 2D)

B. % CCR5+ classical (PRE, ΔPRE - 1D)

C. % IFN-γ+ CM CD4+ T-cells 2D vs % CCR5+ classical PRE

D. % CX3CR1+ non-classical (PRE, 1D, 2D)

E. % IFN-γ+ CM CD4+ T-cells 2D vs % CX3CR1+ non-classical 2D

F. % CD11b+ intermediate (PRE, 1D, 2D)

G. % IFN-γ+ CM CD4+ T-cells 2D vs % CD11b+ classical 2D
Figure 7. A diminished monocyte activation and homing found in aged people is related to a lower SARS-CoV-2 vaccine response

(A) Dot plots representing the percentage of classical monocytes expressing CD40 (top) and TLR-4 (bottom) in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination (left). Correlation matrix representing the percentage of monocytes expressing CD40 and TLR-4 prior to vaccination with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing cytokines or cytotoxicity markers two months after the second dose (right). (B and C) Dot plots representing the percentage of classical monocytes expressing CCR5 before vaccination (B, left), the fold of decrease in the percentage of CCR5+ cells after the first dose (B, right) and correlation analysis of the percentage of CCR5+ classical monocytes prior to vaccination with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing IFN-γ two months after the second dose (C). (D-E) Dot plots showing the percentages of non-classical monocytes expressing CX3CR1 in >60 years old (red) and <60 years old (blue) subjects at the three follow up time points (D). Correlations of the percentage of non-classical monocytes expressing CX3CR1 two months after the second dose (E, left) and prior vaccination (E, right) with SARS-CoV-2 S-specific IFN-γ+ CD4+ T cells after the second dose. (F-G) Dot plots showing the percentages of monocytes expressing CD11b in >60 years old (red) and <60 years old (blue) subjects at the three follow up time points (F). Correlation plots between the percentage of CD11b+ classical monocytes with SARS-CoV-2 S-specific IFN-γ+ CD4+ T cells (G, left) and anti-RBD IgG levels (G, right) two months after the second dose. Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 28). Friedman test was applied in panel A, left (CD40: >60 years old, p= 0.006; <60 years old, p= 0.050 and TLR4: >60 years old, p= 0.042; <60 years old, p= 0.779), D (>60 years old, p= 0.002; <60 years old, p= 0.717) and F (>60 years old, p= 0.607; <60 years old, p= 0.368).
Figure 8. Higher monocyte-mediated proinflammatory profile found in aged people is associated to a lower T-cell response to SARS-CoV-2 vaccine

(A) Bar graphs representing the percentage of classical, intermediate and non-classical monocytes in >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination (right). Pseudocolour plots showing representative data of an >60 years old and <60 years old participant two months after the second dose (left). (B) Box and whiskers graphs representing the percentage of IL-6+, IL-1α+ and TNF-α+ monocytes upon TLR-4 stimulation in >60 years old (red) and <60 years old (blue) participants at the three time points (bottom). Contour plots showing representative data of the percentage of cytokine+ monocytes from an >60 years old and <60 years old subject two months after the second dose (top). (C) Bar graphs showing the percentage of cytokine (IL-6, IL1-α and TNF-α) producing monocytes on individuals with a cytotoxic SARS-CoV-2 S-specific T cell response and the ones with a negative response. The percentage of specific PRF+ T cells higher than 0.01 was considered as a positive cytotoxic T cell response. Mann-Whitney U and Wilcoxon tests were used (n= 26). Friedman test was applied in panel B, bottom (IL-6: >60 years old, p= 0.223; <60 years old, p= 0.368, IL-1α: >60 years old, p= 1.00; <60 years old, p= 0.368 and TNF-α: >60 years old, p= 0.223; <60 years old, p= 0.368).