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Age differentially affects the maintenance of adaptive immune responses induced by adenoviral versus mRNA vaccines against COVID-19

Francesco Nicoli
nclfnc1@unife.it
University of Ferrara https://orcid.org/0000-0001-6327-5958
Beatrice Dallan
University of Ferrara
Davide Proietto
University of Ferrara
Martina De Laurentis
University of Ferrara
Eleonora Gallerani
University of Ferrara
Mara Martino
University of Ferrara
Sara Ghisellini
St. Anna Hospital, Ferrara
Amedeo Zurlo
University of Ferrara
Stefano Volpato
University of Ferrara
Benedetta Govoni
University of Ferrara
Michela Borghesi
University of Ferrara
Valentina Albanese
University of Ferrara https://orcid.org/0000-0002-1947-2644
Victor Appay
Immuconcept CNRS UMR 5164
Stefano Bonnini
University of Ferrara
Sian Llewellyn-Lacey

Cardiff University
Salvatore Pacifico
University of Ferrara
David Price
Cardiff University https://orcid.org/0000-0001-9416-2737
Antonella Caputo
University of Ferrara
Riccardo Gavioli
University of Ferrara

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Abstract

Adenoviral and mRNA vaccines encoding the viral spike protein have been deployed globally to contain SARS-CoV-2. Elderly individuals are particularly vulnerable to severe infection, likely reflecting age-related changes in the immune system, which can also compromise vaccine efficacy. It has nonetheless remained unclear to what extent different vaccine platforms are impacted by immunosenescence. Here, we evaluated spike-specific immune responses elicited by vaccination with two doses of BNT162b2 or ChAdOx1-S and subsequently boosted with a single dose of BNT162b2 or mRNA-1273, comparing age-stratified participants with no evidence of prior infection with SARS-CoV-2. We found that ageing profoundly affected the durability of humoral responses and further limited spike-specific $CD4^+$ T cell immunity as a function of progressive erosion of the naive lymphocyte pool in individuals vaccinated initially with BNT162b2, such that protective immunological memory was best maintained in the elderly after primary vaccination with ChAdOx1-S and subsequent boosting with BNT162b2 or mRNA-1273.

One Sentence Summary

SARS-CoV-2-specific vaccine-induced adaptive immunity is optimally maintained in the elderly after primary immunization with ChAdOx1-S and subsequent boosting with BNT162b2 or mRNA-1273.

INTRODUCTION

Immunization against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) began at the end of 2020, preventing millions of deaths worldwide [1]. The first approved vaccines were based on mRNA technology, namely BNT162b2 (BioNTech/Pfizer) or mRNA-1273 (Moderna), or adenoviral vectors, namely ChAdOx1-S (AstraZeneca) or Ad26.COV2.S (Janssen), encoding the spike protein of SARS-CoV-2. However, it subsequently became clear that vaccine efficacy waned quickly [2,3], especially against newly emerging viral variants that diverged rapidly from the wildtype strain [4–8]. The global spread of variants of concern (VOCs) that could escape existing immunity [8] led to the introduction of further booster immunizations beyond the third dose to maintain vaccine efficacy, which had already exceeded 90% [9–11]. National regulations dictated which vaccine formulation was used for boosting after the second dose [12].

As the virus spread globally, it became clear that elderly individuals were particularly susceptible to severe disease, with high attendant rates of mortality [13–16]. Vaccine efficacy was also found to be suboptimal in the elderly [17,18], facilitating breakthrough infections with SARS-CoV-2 [19,20]. These observations suggested an age-related link between antiviral immunity and disease outcome. Physiological ageing has been linked with a progressive decline in immune functionality, known as immunosenescence, which could account for the increased vulnerability of elderly individuals to coronavirus disease 2019 (COVID-19) [21,22]. Accordingly, we recently demonstrated that age is associated with a reduction in the diversity and magnitude of functional $CD8^+$ T cell responses elicited against SARS-CoV-2 [23,24], consistent with the typical features of immunosenescence [25–27], which collectively

limit the ability of elderly individuals to mount *de novo* adaptive immune responses against previously unencountered pathogens in the context of natural infection and/or vaccination [28,29]. This phenomenon impacts both cellular and humoral immunity [30]. In line with these observations, recent studies have shown that the BNT162b2 vaccine becomes less effective with age [2,31–33], as reported previously for vaccines against other viral infections, such as yellow fever and hepatitis B [34–37]. To circumvent this issue, booster doses of some vaccines are recommended throughout life [38], but this solution is only partially effective in the elderly [39].

SARS-CoV-2 vaccine efficacy has been shown to correlate with antibody-mediated neutralization, which is generally directed against the receptor-binding domain (RBD) of the spike protein [40–43]. Studies to date have indicated that neutralizing antibody titers decline rapidly within a few months after vaccination [42,43], whereas spike-specific $CD4^+$ and $CD8^+$ T cell-mediated immunity appears to be somewhat more stable [44,45]. However, the extent to which age and the type of vaccine affect the durability of cellular and humoral immune responses has not been addressed directly. In this study, we characterized cellular and humoral immunity as a function of age at memory time points after primary vaccination with two doses of BNT162b2 or two doses of ChAdOx1-S and after booster vaccination with a subsequent dose of BNT162b2 or mRNA-1273. Our data revealed that age negatively impacted immunological memory more profoundly after primary vaccination with BNT162b2 versus ChAdOx1-S, irrespective of subsequent boosting with BNT162b2 or mRNA-1273.

RESULTS

ChAdOx1-S is less reactogenic than BNT162b2

To assess the durability of vaccine-induced adaptive immunity against SARS-CoV-2, we measured spike-specific cellular and humoral responses in young (Y, 18–40 years), middle-aged (M, 41–65 years), and old individuals (O, > 65 years) at a median of 6 months (range, 4– 10 months) after the second of two doses of BNT162b2 (n = 131) or two doses of ChAdOx1-S (n = 93) and at a median of 6 months (range, 4–9 months) after a booster dose of BNT162b2 or mRNA-1273 following primary immunization with BNT162b2 (n = 79) or ChAdOx1-S (n = 59) (Fig. 1 and Supplementary Figure S1). Comorbidities and demographics are reported in Supplementary Tables S1 and S2. Side effects after vaccination were generally milder in group O compared with groups Y and M (Table 1). Moreover, the second and third doses were less reactogenic across all age groups vaccinated initially with ChAdOx1-S, and side effects were more protracted among individuals vaccinated initially with BNT162b2, especially in group O.

Table 1
Distribution of side effects after vaccination stratified by age.

Donors vaccinated with two doses of ChAdOx1-S (*AstraZeneca*) and boosted with one dose of BNT162b2 (*Pfizer/BioNTech*) or mRNA-1273 (*Moderna*)									
	I dose			II dose			III dose		
	Y (26)	M (69)	O (10)	Y (26)	M (69)	O (10)	Y (11)	M (40)	O (8)
Side effects									
Pain/swelling (%)	46	42	0	35	35	0	55	35	13
Fatigue (%)	62	62	30	35	30	30	27	28	25
Headache (%)	50	38	10	23	17	10	9	3	0
Fever (%)	65	42	20	19	6	20	18	8	0
Muscle/joint pain (%)	46	33	30	23	7	20	18	8	0
Diarrhea (%)	0	1	0	0	1	0	0	0	0
Nausea (%)	8	3	0	0	0	0	9	0	0
Allergic reaction (%)	0	0	0	0	0	0	9	0	0
Blood clotting (%)	0	1	0	0	0	0	0	0	0
Facial paralysis (%)	0	0	0	0	0	0	0	0	0
Asymptomatic (%)	8	9	40	46	41	50	36	40	63
Duration									
24 h (%)	58	65	30	35	52	50	46	45	25
2–3 days (%)	23	20	20	19	4	0	18	15	0
1 week (%)	11	4	10	0	3	0	0	0	12
> 1 week (%)	0	2	0	0	0	0	0	0	0
Donors vaccinated with two doses of BNT162b2 (Pfizer/BioNTech) and boosted with one dose of BNT162b2 (Pfizer/BioNTech) or mRNA-1273 (Moderna)									
	I dose			II dose			III dose		
	Y	M	O	Y	M	O	Y	M	O

Donor numbers are indicated in parentheses: Y, young (18–40 years); M, middle-aged (41–65 years); O, old (> 65 years). [1]Data from 65 donors. [2]Data from 58 donors. [3]Data from 66 donors. [4]Data from 64 donors. [5]Data from 65 donors.

Donors vaccinated with two doses of ChAdOx1-S (*AstraZeneca*) and boosted with one dose of BNT162b2 (*Pfizer/BioNTech*) or mRNA-1273 (*Moderna*)									
	(67)	(59)	(38)	(67)	(59)	(38)	(16)	(35)	(28)
Side effects									
Pain/swelling (%)	74[1]	71[2]	68	73[3]	63	71	44	63	71
Fatigue (%)	46[1]	21[2]	13	56[3]	42	13	50	31	11
Headache (%)	18[1]	16[2]	3	27[3]	22	0	13	11	0
Fever (%)	8[1]	2[2]	3	33[3]	20	3	38	14	0
Muscle/joint pain (%)	22[1]	10[2]	5	23[3]	24	0	31	11	4
Diarrhea (%)	2[1]	2[2]	0	0[3]	3	0	0	3	0
Nausea (%)	0[1]	2[2]	0	5[3]	7	0	0	6	0
Allergic reaction (%)	0[1]	3[2]	0	0[3]	2	0	0	0	0
Blood clotting (%)	0[1]	0[2]	0	0[3]	0	0	0	0	0
Facial paralysis (%)	0[1]	0[2]	0	0[3]	0	0	0	0	0
Asymptomatic (%)	12[1]	17[2]	31	9[3]	12	26	12	23	25
Duration									
24 h (%)	55[4]	43[2]	13	61[5]	46	16	76	46	14
2–3 days (%)	31[4]	33[2]	53	28[5]	32	58	12	28	61
1 week (%)	2[4]	2[2]	3	2[5]	7	0	0	3	0
> 1 week (%)	0[4]	5[2]	0	0[5]	3	0	0	0	0

Donor numbers are indicated in parentheses: Y, young (18–40 years); M, middle-aged (41–65 years); O, old (> 65 years). [1]Data from 65 donors. [2]Data from 58 donors. [3]Data from 66 donors. [4]Data from 64 donors. [5]Data from 65 donors.

Age impacts cellular and humoral immune responses elicited by primary vaccination with BNT162b2

We first evaluated vaccine-induced immunity as a function of age. RBD-specific antibodies were almost absent (< 10AU/ml) after two doses of ChAdOx1-S, irrespective of age, whereas the booster dose induced long-lasting RBD-specific antibodies in an age-independent fashion (Fig. 2A). In contrast,

vaccination with BNT162b2, either without or with the booster dose, induced higher titers of RBD-specific antibodies in younger participants, evidenced by consistent inverse correlations between age and RBD-specific IgG titers and by a progressive decrease in responder frequencies across groups Y, M, and O (Fig. 2A). A similar pattern was observed for $CD8^+$ T cells specific for the immundominant HLA-A2-restricted spike epitope YLQPRTFLL (YLQ, residues 269–277), as assessed via tetramer staining after transient expansion, which decreased as a function of age after primary vaccination with BNT162b2 but not after primary vaccination with ChAdOx1-S (Figs. 2B and C). These cells were nonetheless able to mediate cytotoxic activity after further expansion from individuals in groups M and O (Fig. 2D).

No significant age-related differences in magnitude were detected for $CD4^+$ T cell responses against the whole spike protein of SARS-CoV-2 after primary vaccination, irrespective of the formulation, as assessed via intracellular cytokine staining after transient expansion in the presence of overlapping 15mer peptides spanning the entire protein and after recall stimulation, as assessed in terms of CD107a mobilization, an indicator of degranulation, and the production of interferon (IFN)γ and tumor necrosis factor (TNF) (Fig. 3A–C). However, a direct correlation with age was observed for $IFN\gamma^+$ $CD4^+$ T cell frequencies elicited by the booster dose after primary vaccination with ChAdOx1-S, whereas an inverse correlation with age was observed for $IFN\gamma^+$ $CD4^+$ T cell frequencies elicited by the booster dose after primary vaccination with BNT162b2 (Fig. 3B). This latter finding was paralleled by an age-related decrease in responder frequencies across all functions, namely CD107a, IFNγ, and TNF (Fig. 3A–C). Spike-specific $CD4^+$ T cells also became less polyfunctional as a function of age after the booster dose, irrespective of the primary vaccine formulation (Fig. 3D). In contrast, no significant age-related differences in magnitude were detected for $CD8^+$ T cell responses against the whole spike protein of SARS-CoV-2 after vaccination, either with or without the booster dose (Figs. 3E–G). Responder frequencies measured in terms of IFNγ or TNF production were nonetheless somewhat lower after primary vaccination with BNT162b2 and subsequent boosting in group O compared with groups Y and M (Fig. 3F, G). The booster dose also enhanced the polyfunctionality of spike-specific $CD8^+$ T cells after primary vaccination with ChAdOx1-S in groups M and O relative to group Y but reduced the polyfunctionality of spike-specific $CD8^+$ T cells after primary vaccination with BNT162b2 in group O relative to groups Y and M (Fig. 3H).

Collectively, these findings indicated that age profoundly affected the durability of humoral responses after vaccination with BNT162b2, either with or without the booster dose, and further limited the maintenance of functional spike-specific $CD4^+$ T cells after the booster dose in individuals vaccinated initially with BNT162b2, such that adaptive immunological memory was best maintained in elderly participants after primary vaccination with ChAdOx1-S and subsequent boosting with BNT162b2 or mRNA-1273.

Immunosenescence curtails immune responses elicited by mRNA vaccination against SARS-CoV-2

To investigate the relationship between cellular and humoral responses after immunization against SARS-CoV-2, we correlated the various measures of adaptive immunity in donors vaccinated initially with BNT162b2 or ChAdOx1-S. Memory $CD4^+$ and $CD8^+$ T cell frequencies were largely independent of each other and RBD-specific IgG titers after two doses of BNT162b2 or ChAdOx1-S (Fig. 4A). A similar pattern was observed after the booster dose in donors vaccinated initially with ChAdOx1-S (Fig. 4A). In contrast, almost all measures of adaptive immunity correlated directly with each other after the booster dose in donors vaccinated initially with BNT162b2, indicating a coordinated response (Fig. 4A).

In all cases, it seemed likely that the observed antiviral immune responses stemmed primarily from naive B and T cells, which became less frequent with age (Supplementary Figure S2), given that we only recruited participants with no history of infection with SARS-CoV-2. Although we found no consistent associations linking the absolute numbers of naive B cells or naive $CD4^+$ or $CD8^+$ T cells with RBD-specific IgG titers or the frequencies of spike-specific $CD4^+$ or $CD8^+$ T cells across vaccine formulations, direct correlations were apparent between the absolute numbers of naive $CD4^+$ T cells and the frequencies of spike-specific $CD4^+$ and $CD8^+$ T cells after the booster dose in donors vaccinated initially with BNT162b2 (Fig. 4B). In line with these observations, which suggested a central role for $CD4^+$ T cells in the induction and/or maintenance of cellular immune responses after mRNA vaccination, spike-specific $CD4^+$ and $CD8^+$ T cell responses were more balanced after primary immunization with BNT162b2 versus ChAdOx1-S. Indeed ChAdOx1-S predominantly elicited spike-specific $CD8^+$ T cell responses that remained prevalent after the booster dose (Fig. 4C). It was also notable that RBD-specific IgG titers correlated directly with the absolute numbers of naive B cells and $CD8^+$ T cells after the booster dose in donors vaccinated initially with BNT162b2 but not in donors vaccinated initially with ChAdOx1-S (Fig. 4B).

Collectively, these observations indicated that age-related erosion of the naive lymphocyte pool, a typical feature of immunosenescence, constrained the induction and/or maintenance of adaptive immune responses elicited by the booster dose after primary vaccination with BNT162b2 but not after primary vaccination with ChAdOx1-S.

Comorbidities and cytomegalovirus limit immune responses after primary vaccination with BNT162b2

To investigate other factors associated with vaccine immunogenicity, we stratified participants as responders versus nonresponders for RBD-specific IgG titers (positive, > 10 AU/ml) and as responders versus nonresponders or poor responders for the frequencies of spike-specific $CD4^+$ (positive, > 0.2%) and $CD8^+$ T cells (positive, > 0.4%). No obvious associations with RBD-specific IgG titers were identified across multiple parameters, including age, various comorbidities, cytomegalovirus (CMV) serostatus [28], and gender after primary vaccination with ChAdOx1-S, either with or without the booster dose (Fig. 5A). In contrast, old age, the presence of multiple comorbidities, and CMV seropositivity were associated with a lack of humoral reactivity in donors vaccinated initially with BNT162b2, irrespective of the booster dose

(Fig. 5A). Mild or nonexistent side effects were also associated with humoral nonresponsiveness after the second dose of BNT162b2, whereas physical activity was associated with humoral responsiveness after the second dose of BNT162b2 (Fig. 5A).

A similar pattern was observed for spike-specific $CD4^+$ T cell responses after the booster dose in donors vaccinated initially with BNT162b2 (Fig. 5B). This observation reinforced the notion that helper functions provided by cognate $CD4^+$ T cells were critical for the development of a coordinated immune response after mRNA vaccination. In contrast, only mild or nonexistent side effects after the second or third dose impacted spike-specific $CD8^+$ T cell responses after the booster dose in donors vaccinated initially with BNT162b2, and only a high body mass index impacted spike-specific $CD8^+$ T cell responses after the booster dose in donors vaccinated initially with ChAdOx1-S (Fig. 5C).

Collectively, these data indicated that age, multiple comorbidities, and CMV seropositivity impacted the induction and/or maintenance of humoral and $CD4^+$ T cell responses after primary vaccination with BNT162b2 but not after primary vaccination with ChAdOx1-S.

Age impacts the coordination of adaptive immune responses after vaccination against SARS-CoV-2

To confirm these findings, we identified donors with strong or weak cellular and humoral responses after the second or third vaccine dose (B/T responders or B/T nonresponders, respectively), as defined in Supplementary Figure S3A. A majority of B/T responders after the second dose were immunized with BNT162b2 and were seronegative for CMV (Supplementary Figure S3B), whereas a majority of B/T responders after the booster dose undertook physical activity (Supplementary Figure S3B). After the second dose, B/T responders were younger than B/T nonresponders, and a similar trend was observed after the booster dose (Supplementary Figure S3C). B/T responders also exhibited fewer comorbidities after the third dose compared with B/T nonresponders, albeit below the threshold for significance (Supplementary Figure S3C). The absolute numbers of memory B cells but not naive B cells or plasma cells were higher in B/T responders versus B/T nonresponders (Supplementary Figure S3D), and similar trends were observed for the absolute numbers of naive $CD4^+$ and $CD8^+$ T cells, although significance was achieved only for the latter after the booster dose (Supplementary Figure S3E).

Collectively, these results indicated that age-related factors, including features of immunosenescence, impacted the induction and/or maintenance of coordinated cellular and humoral immune responses after vaccination against SARS-CoV-2.

Cytomegalovirus impacts cellular immune responses after primary vaccination against SARS-CoV-2

Although our data suggested that CMV infection negatively impacted cellular and humoral immune responses elicited by primary vaccination with BNT162b2 (Supplementary Figure S3B), an indirect correlation remained possible, given the increasing prevalence of seropositivity with age (Supplementary

Figure S4A). To address this issue, we performed further analyses focused on donors aged < 50 years, stratified by serostatus for CMV. Higher absolute numbers of differentiated memory $CD4^+$ and $CD8^+$ T cells were present in seropositive versus seronegative donors, whereas the absolute numbers of B cells were comparable (Supplementary Figure S4B). Adaptive immune responses were analyzed after two doses of BNT162b2 or ChAdOx1-S, because relatively few donors recruited after the booster dose were seronegative for CMV. No significant differences in RBD-specific IgG titers were detected among groups stratified by vaccine formulation or serostatus for CMV (Supplementary Figure S4C). However, YLQ-specific $CD8^+$ T cell frequencies were significantly lower in seropositive versus seronegative donors, achieving significance after primary vaccination with BNT162b2 (Fig. 5D), whereas spike-specific $CD4^+$ but not $CD8^+$ T cell frequencies were significantly lower in seropositive versus seronegative donors after primary vaccination with ChAdOx1-S (Fig. 5E).

Collectively, these findings indicated that cellular but not humoral responses were adversely affected by infection with CMV, primarily impacting $CD4^+$ T cell immunity in donors vaccinated initially with ChAdOx1-S and $CD8^+$ T cell immunity in donors vaccinated initially with BNT162b2.

Age minimally impacts the diversity of $CD8^+$ T cell responses elicited by vaccination against SARS-CoV-2

To evaluate the impact of age on memory $CD8^+$ T cell specificities, we performed IFNγ ELISpot assays directly *ex vivo* using HLA-A2-restricted peptides representing optimally defined SARS-CoV-2 spike epitopes (Supplementary Table S3) and, for control purposes, a pool of commonly recognized HLA-A2-restricted peptides (MEM) representing optimally defined epitopes from influenza virus (Flu), Epstein-Barr virus (EBV), and herpes simplex virus (HSV) (Supplementary Table S4). No obvious differences in magnitude as a function of age or vaccination schedule were apparent for $CD8^+$ T cell responses targeting the SARS-CoV-2 spike peptides, but MEM-specific $CD8^+$ T cell responses were generally less prominent in older versus younger participants, irrespective of primary immunization with BNT162b2 or ChAdOx1-S (Fig. 6A). Comparable immunoprevalence patterns were also observed across the SARS-CoV-2 spike peptides as a function of age, irrespective of the vaccination schedule, although the KIA peptide was more commonly recognized by individuals in group Y relative to groups M and O, and the VVF peptide was less commonly recognized by individuals in group O relative to groups Y and M (Fig. 6B).

Collectively, these observations indicated that spike-specific $CD8^+$ T cell responses remained rather intact in the elderly after primary vaccination with BNT162b2 or ChAdOx1-S.

DISCUSSION

COVID-19 vaccines become less effective over time in terms of preventing infection and symptomatic disease [3], likely as a consequence of waning antibody-mediated neutralization [46], whereas protection

against severe disease appears to be maintained more durably [47], likely as a consequence of cell-mediated immunological memory [48], which is generally more stable [49]. It has nonetheless remained unclear to what extent age-related changes in the immune system impact the efficacy and immunogenicity of vaccines designed to elicit protective immunity against SARS-CoV-2. To address this issue, we performed a comprehensive evaluation of spike-specific adaptive immune responses as a function of age after primary vaccination with two doses of BNT162b2 or ChAdOx1-S and subsequent boosting with a single dose of BNT162b2 or mRNA-1273, excluding donors with evidence of prior infection with SARS-CoV-2. We found that ageing profoundly affected the durability of spike-specific humoral responses and notably compromised the maintenance of spike-specific $CD4^+$ T cell responses as a function of progressive erosion of the naive lymphocyte pool in donors vaccinated initially with BNT162b2. These collective findings indicated that adaptive immunological memory was optimally maintained in elderly individuals after primary vaccination with ChAdOx1-S and subsequent boosting with BNT162b2 or mRNA-1273.

A key finding of the present study was that age impacted the maintenance of humoral responses after primary vaccination with BNT162b2 but not after primary vaccination with ChAdOx1-S. It has been shown previously that spike-specific antibody titers are higher in younger versus older recipients even a few days after primary vaccination with BNT162b2 [2,32,50–52], whereas no such age-related effects have been observed after primary vaccination with ChAdOx1-S [31]. Our results indicated that a similar dichotomy applied at memory time points, consistent with an intrinsic difference in humoral immune kinetics that segregated with the primary vaccine formulation [44,53].

We show here that memory cellular responses are lost, with advancing age, in BNT162b2- but not in ChAdOx1-S-recipients. Consistently, age has been shown to impair spike-specific cellular immune responses after primary vaccination with BNT162b2 but not after primary vaccination with ChAdOx1-S [32,33,54,55]. Our results indicated that a similar phenomenon extended into memory, and primarily impacted the maintenance of spike-specific $CD4^+$ T cell immunity [54,56]. Instead, the secretory capacity of spike-specific $CD8^+$ T cells was maintained in older subjects irrespective of the formulation, while we observed major defects in the recall expansion of epitope-specific $CD8^+$ T cells generated in older subjects upon vaccination with BNT162b2. In addition, age slightly reduced the responses to certain epitopes, consistently with previous studies showing a narrower epitope-specific repertoire in spike-specific naive $CD8^+$ T cells from unexposed and unvaccinated older individuals [23,24].

Our results indicated that BNT162b vaccination induced a well-coordinated cellular and humoral response, while ChAdOx1-S preferentially elicited $CD8^+$ than $CD4^+$ responses and mutual independent cellular and humoral immunities. The lower memory responses in older subjects vaccinated with BNT162b were linked via associations with age-related factors, such as multiple comorbidities, physical inactivity, and seropositivity for CMV, and features of immunosenescence, most notably the absolute numbers of naive $CD4^+$ T cells, consistent with the development of a uniquely coordinated memory response after primary vaccination with BNT162b. Therefore, and given that mRNA vaccines have

previously shown to predominantly induce T helper cells that patrol the whole vaccine-induced immunity [42,49], we could speculate that the immune responses elicited by this vaccine type are more sensitive to age-related alterations, especially at the level of the $CD4^+$ compartment, impacting both memory T helper and humoral responses. Conversely, vectored vaccines can probably overcome, at least in part, major immunosenescence features through molecular mechanism that deserve further investigations.

Due to the very low frequency of CMV-negative subjects in volunteers older than 50 years, we could not assess the direct effect of CMV infection on memory responses from aged individuals, which has been previously shown to be marginal in the context of COVID-19 vaccines [57]. Conversely, when focusing on subjects < 50 years old, we observed lower cellular, but not humoral, responses in CMV-positive individuals, especially in respect to $CD4^+$ immunity, in agreement with previous findings suggesting a major impact of CMV on this harm of the adaptive immunity [28].

Collectively, we found that age profoundly affected the durability of humoral responses after vaccination with BNT162b2, either with or without the booster dose, and further limited the maintenance of functional spike-specific $CD4^+$ T cell responses after the booster dose in individuals vaccinated initially with BNT162b2, likely explaining why vaccine efficacy measured in terms of hospitalization declines over time in the elderly after primary vaccination with BNT162b2 [18,58] but not after primary vaccination with ChAdOx1-S [58].

METHODS

Study cohort

Healthy donors with no known history of SARS-CoV-2 infection ($n = 244$) were enrolled at a median of 6 months (range, 4–10 months) after a second homologous immunization against COVID-19, comprising either two doses of BNT162b2 ($n = 131$) or two doses of ChAdOx1 ($n = 93$) (Supplementary Table S1). A subset of these participants ($n = 93$) and newly enrolled volunteers ($n = 45$) also provided samples at a median of 6 months (range, 4–9 months) after boosting with BNT162b2 or mRNA-1273 (Supplementary Table S2). Donors in all groups were stratified by age as young (Y, 18–40 years), middle-aged (M, 41–65 years), or old (O, > 65 years). The study was approved by the Ethical Committee of the Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani (protocol number 488). All participants provided written informed consent in accordance with the principles of the Declaration of Helsinki. Venous blood samples were collected into acid citrate dextrose tubes from donors recruited at long-term care facilities, the Ferrara Blood Bank, or the Geriatric Department of Ferrara Hospital, Italy.

Samples and serology

Peripheral blood mononuclear cells (PBMCs) were isolated via standard density gradient centrifugation using Ficoll-Paque (GE Healthcare), suspended in 90% fetal bovine serum (FBS, Euroclone) and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich), and cryopreserved in liquid nitrogen. Plasma samples were

stored at – 80°C. SARS-CoV-2 spike RBD-specific IgG titers were determined using an Access SARS-CoV-2 IgG II Assay (Beckman Coulter). IgG titers were considered positive at > 10 AU/ml. CMV serology was determined using a LIAISON CMV IgG II Kit (DiaSori).

Peptides and tetramers

HLA-A2-restricted peptides representing optimally defined SARS-CoV-2 spike epitopes (Supplementary Table S3) were used to assess memory $CD8^+$ T cell responses, alongside a pool of commonly recognized HLA-A2-restricted peptides (MEM) representing optimally defined epitopes from Flu, EBV, and HSV (Supplementary Table S4). All peptides were synthesized in solid phase and purified via reversed-phase high-performance liquid chromatography to > 97%. SARS-CoV-2 peptides were selected from the wildtype spike protein (strain HKU-001a) on the basis of immunodominance and immunoprevalence [23]. SARS-CoV-2 spike 15mer overlapping peptides (n = 181) were obtained from BEI Resources (NR-52402). Individual or pooled peptides were suspended in DMSO and used at a final concentration of 1 µM. The fluorescent peptide/HLA-A2 tetramer corresponding to the YLQ epitope was generated as described previously [59].

ELISpot assay

$CD8^+$ T cell responses were enumerated directly *ex vivo* using a Human IFNγ ELISpot PLUS (HRP) Kit (Mabtech). PBMCs were seeded in duplicate at 2.5×10^5 cells/well in precoated capture plates and stimulated with individual peptides or the MEM pool for 24 h. Negative control wells lacked peptide, and positive control wells contained anti-CD3 (clone CD3-2, Mabtech). Plates were developed according to the manufacturer’s instructions and analyzed using an automated ELISpot Reader (AELVIS). IFNγ-secreting cells were quantified as spot-forming units (SFUs) per 10^6 cells after background subtraction. Results were excluded if the positive control was negative. Responses were considered positive at > 10 SFUs/10^6 PBMCs.

Flow cytometry

Immunophenotypic analyses were performed using whole fresh blood stained with anti-CD4–PE-Cy7 (clone RM4-5, Thermo Fisher Scientific), anti-CD8–FITC (clone MEM-31, ImmunoTools), anti-CD19–VioGreen (clone REA675, Miltenyi Biotec), anti-CD27–APC (clone REA499, Miltenyi Biotec), anti-CD38–VioBlue (clone REA671, Miltenyi Biotec), anti-CD45RA–PerCP-Cy5.5 (clone HI100, Thermo Fisher Scientific), and anti-HLA-A2–PE (clone BB7.2, BioLegend). Red blood cells were lysed using FACS Lysis Solution (BD Biosciences). Functional analyses were performed after *in vitro* expansion on day 10. Cells were stimulated with or without SARS-CoV-2 spike 15mer overlapping peptides for 6 h in the presence of anti-CD107a–VioBlue (clone REA792, Miltenyi Biotec). Brefeldin A (2 µg/ml, Sigma-Aldrich) and monensin (2 µM, Thermo Fisher Scientific) were added after 1 h. After stimulation, cells were labeled with LIVE/DEAD Fixable Aqua (Thermo Fisher Scientific) and stained with anti-CD3–PerCP-Cy5.5 (clone OKT3, Thermo Fisher Scientific), anti-CD4–PE-Cy7 (clone RM4-5, Thermo Fisher Scientific), and anti-CD8–APC/Fire 750 (clone SK1, BioLegend). Cells were then fixed/permeabilized using BD

Cytofix/Cytoperm (BD Biosciences) and stained with anti-IFNγ–FITC (clone 4S.B3, Thermo Fisher Scientific), anti-TNF–PE (clone Mab11, BD Biosciences), and anti-CD154–APC (clone 24–31, Thermo Fisher Scientific). Boolean gating was performed for $CD107a^+$, $IFN\gamma^+$, and TNF^+ events among viable $CD3^+CD4^+CD154^+$ and $CD3^+CD8^+$ populations. Cytokine responses in the absence of peptide stimulation were subtracted individually. Tetramer staining was also performed after *in vitro* expansion on day 10. Cells were labeled with LIVE/DEAD Fixable Aqua (Thermo Fisher Scientific) and stained with the YLQ tetramer as described previously [59] followed by anti-CD3–PerCP-Cy5.5 (clone OKT3, Thermo Fisher Scientific), anti-CD4–FITC (clone OKT4, BD Biosciences), anti-CD8–APC/Fire 750 (clone SK1, BioLegend), anti-CD14–FITC (clone 18D11, ImmunoTools), and anti-CD19–FITC (clone ID3, ImmunoTools). Viable tetramer-labeled $CD3^+CD8^+$ T cells were identified after excluding $FITC^+$ events (dump channel). Data were acquired using a FACS Canto II (BD Biosciences), compensated using BD CompBeads (BD Biosciences), and analyzed using FlowJo version 10.8 (FlowJo LLC).

In vitro expansion of antigen-specific T cells

Spike-specific memory $CD4^+$ and $CD8^+$ T cells were expanded from thawed PBMCs. Briefly, cells from HLA-$A2^+$ donors were cultured with the YLQ peptide (1 μM) in the presence of IL-2 (20 U/ml, Miltenyi Biotec), and cells from all donors were cultured with SARS-CoV-2 spike 15mer overlapping peptides in the presence of IL-2 (20 U/ml, Miltenyi Biotec). Medium was replaced on days 4 and 7 with fresh RMPI 1640 (Euroclone) enriched with 10% FBS (Euroclone), nonessential amino acids (1X, Euroclone), penicillin/streptomycin (1%, Euroclone), sodium pyruvate (1 mM, Sigma-Aldrich), and IL-2 (20 U/ml, Miltenyi Biotec). YLQ-specific $CD8^+$ T cells were analyzed via tetramer staining on day 10. Responses were considered positive at > 0.01%. Spike-specific $CD4^+$ and $CD8^+$ T cells were analyzed via intracellular cytokine staining on day 10 [60]. Responses were considered positive at > 0.05%. Odds ratio calculations were delineated by total $CD4^+$ or $CD8^+$ T cell responses > 0.2% or > 0.4%, respectively.

Cytotoxicity assay

PBMCs from HLA-$A2^+$ donors were thawed and plated at 10^6 cells/well in 48-well tissue culture plates (Thermo Fisher Scientific). Cells were stimulated with the YLQ peptide in the presence of IL-2 (20 U/ml, Miltenyi Biotec) and IL-7 (20 ng/ml, R&D Systems) added on days 1, 8, 15, and 22. Medium was replaced on days 4, 11, and 18 with fresh RMPI 1640 (Euroclone) enriched with 10% FBS (Euroclone), nonessential amino acids (1X, Euroclone), penicillin/streptomycin (1%, Euroclone), sodium pyruvate (1 mM, Sigma-Aldrich), IL-2 (20 U/ml, Miltenyi Biotec), and IL-7 (20 ng/ml, R&D Systems). Epitope-specific $CD8^+$ T cells were analyzed via tetramer staining on day 24. $CD8^+$ T cells were then purified via magnetic separation using MS Columns (Miltenyi Biotec). In parallel, an HLA-$A2^+$ lymphoblastoid cell line (LCL) was pulsed with the YLQ peptide (1 μM) for 90 min at 37°C. Pulsed and unpulsed LCLs were then labeled with carboxyfluoroscein succinimidyl ester (CFSE, Thermo Fisher Scientific) and Cell Proliferation Dye eFluor 450 (CPD, Thermo Fisher Scientific), respectively. Assays were set up to contain equal numbers of epitope-specific $CD8^+$ T cells (effectors) and LCLs (targets) and then incubated overnight at 37°C.

Negative control wells lacking effectors were used to calculate background death. Specific killing was calculated as a function of percent survival after background subtraction, comparing the ratio of CFSE-labeled versus CPD-labeled LCLs.

Statistics

Significance was assessed using the Mann-Whitney U test with Bonferroni correction, a one-way ANOVA with Tukey's test, Fisher's exact test, Spearman's rank test, or the Wilcoxon matched-pairs signed-rank test in Prism software version 8 (GraphPad).

Declarations

COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data are available in the main text or supplementary materials.

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Figures

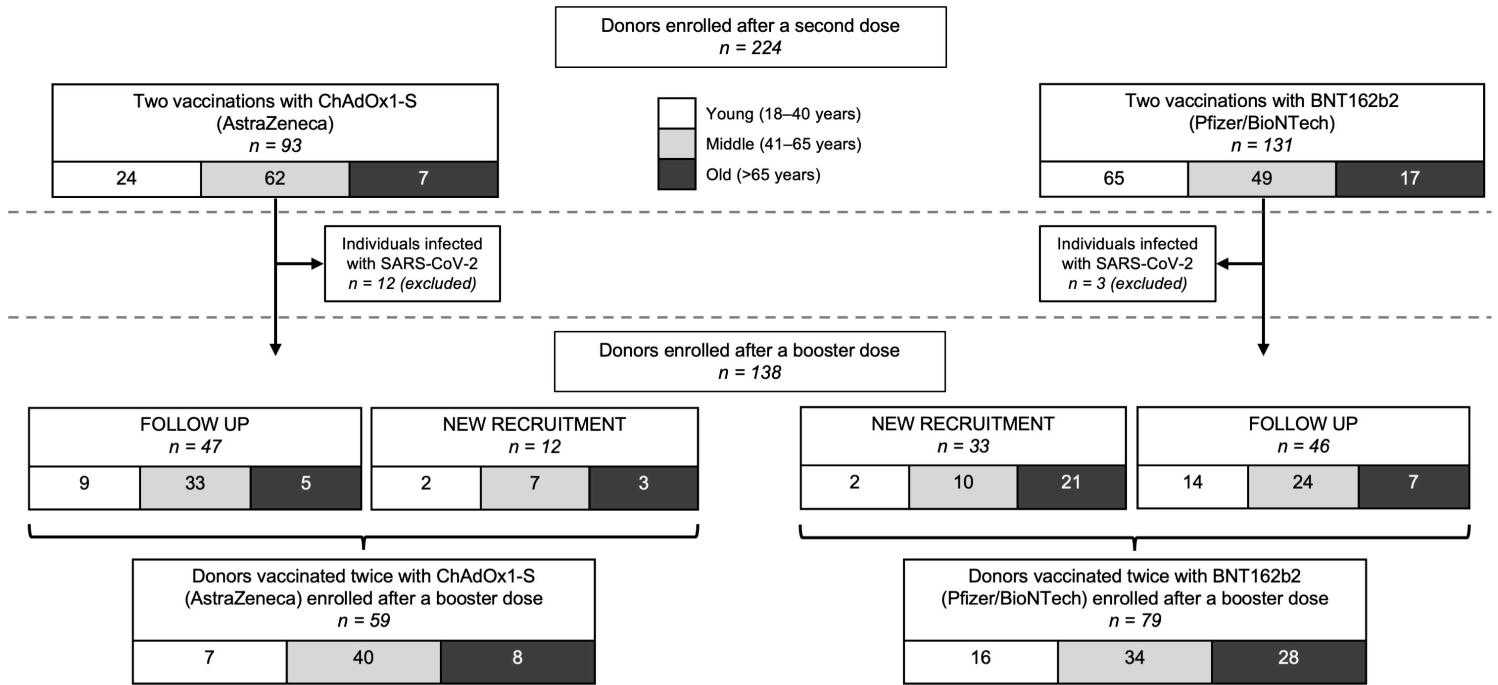


Figure 1

Cohort overview. Participants were enrolled at memory time points (median, 6 months) after a second or third vaccine dose. The booster schedule is summarized in Supplementary Figure S1.

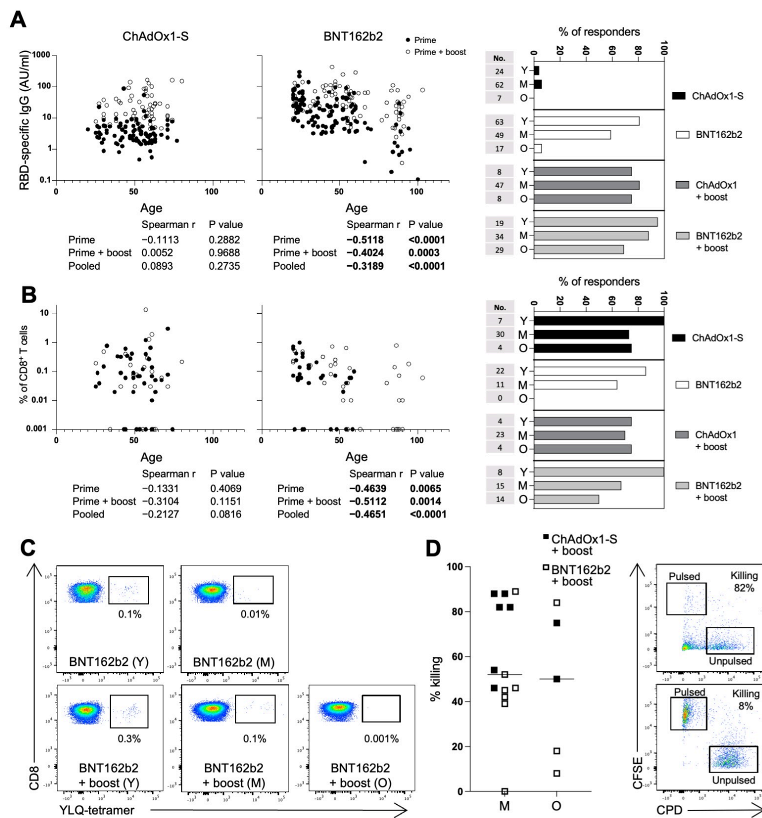


Figure 2

Figure 2

Cytotoxic and humoral immune responses as a function of age after vaccination against SARS-CoV-2.
(**A**) Left: correlations between age and RBD-specific IgG titers (ChAdOx1-S, *n* = 93; BNT162b2, *n* = 129; ChAdOx1-S + boost, *n* = 59; BNT162b2 + boost, *n* = 79). Right: responder frequencies as a function of age (threshold = 10 AU/ml). (**B**) Left: correlations between age and YLQ-specific $CD8^+$ T cell frequencies measured via tetramer staining after transient expansion (ChAdOx1-S, *n* = 41; BNT162b2, *n* = 33; ChAdOx1-S + boost, *n* = 27; BNT162b2 + boost, *n* = 36). Right: responder frequencies as a function of age (threshold = 0.01% of $CD8^+$ T cells). (**C**) Representative flow cytometry plots showing the enumeration of tetramer-labeled YLQ-specific $CD8^+$ T cells as a function of age. Plots are gated on viable $CD3^+$ events after dump exclusion. (**D**) Cytotoxic activity of YLQ-specific $CD8^+$ T cells among donors from groups M (*n* = 12) and O (*n* = 5) after boosting with BNT162b2 or mRNA-1273. Right: representative flow cytometry plots showing the elimination of HLA-$A2^+$ target cells pulsed with YLQ. Correlations were determined using Spearman's rank test (A, B). Horizontal lines represent median values (D).

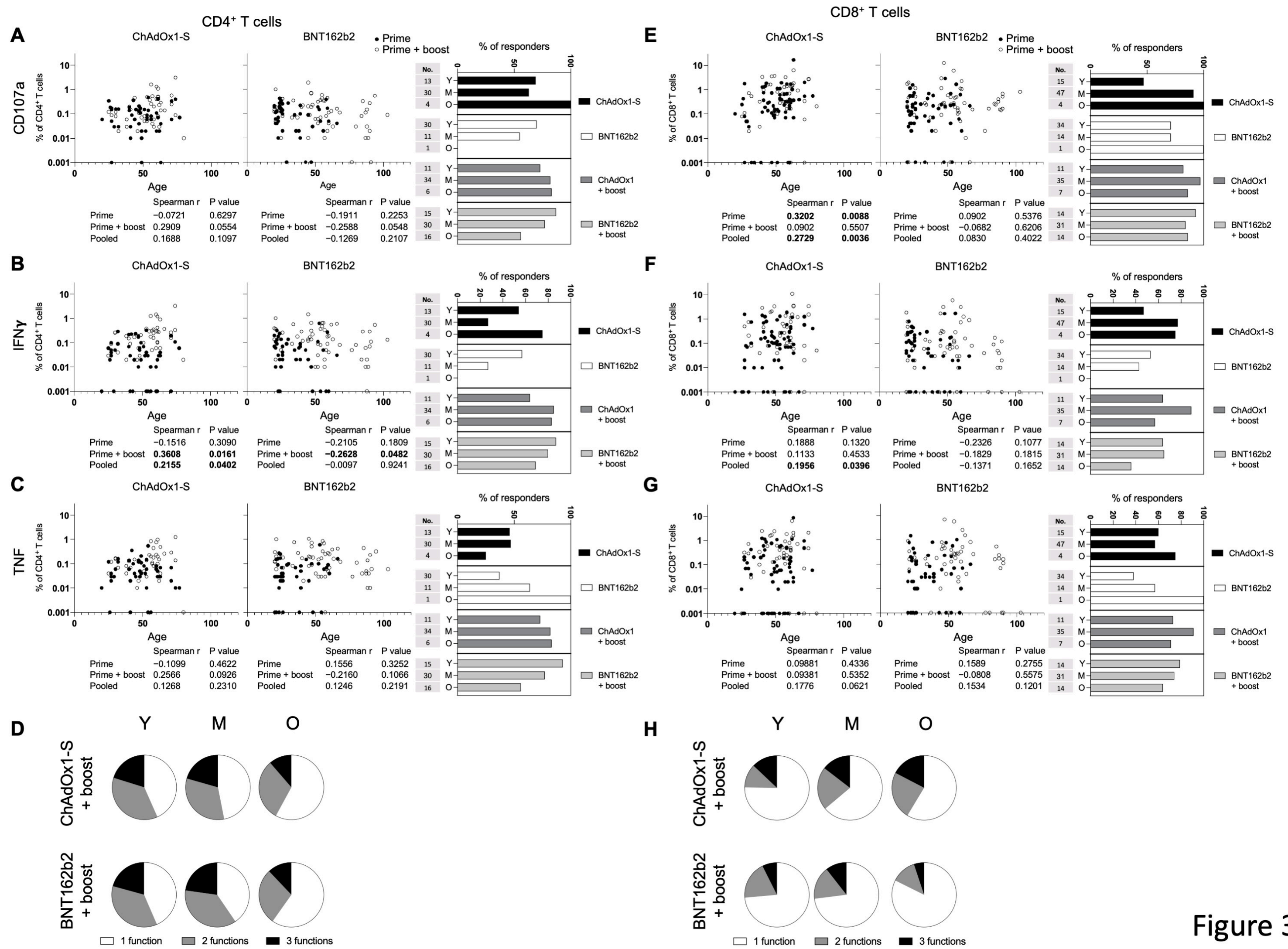


Figure 3

Spike-specific CD4$^+$ and CD8$^+$ T cell responses as a function of age after vaccination against SARS-CoV-2. (**A**–**C**) Left: correlations between age and spike-specific CD4$^+$ T cell frequencies (ChAdOx1-S, *n* = 47; BNT162b2, *n* = 47; ChAdOx1-S + boost, *n* = 44; BNT162b2 + boost, *n* = 59) measured via the recall induction of CD107a (A), IFNγ (B), or TNF (C) after transient expansion. Right: responder frequencies as a function of age (threshold = 0.05% of CD4$^+$ T cells). (**D**) Concatenated functional profiles of spike-specific CD4$^+$ T cells measured as in (A–C) after boosting with BNT162b2 or mRNA-1273. Each pie slice represents the mean number of functions per condition as indicated in the key. (**E**–**G**) Left: correlations between age and spike-specific CD8$^+$ T cell frequencies (ChAdOx1-S, *n* = 65–66; BNT162b2, *n* = 49; ChAdOx1-S + boost, *n* = 46; BNT162b2 + boost, *n* = 57) measured via the recall induction of CD107a (E), IFNγ (F), or TNF (G) after transient expansion. Right: responder frequencies as a function of age (threshold = 0.05% of CD8$^+$ T cells). (**H**) Concatenated functional profiles of spike-specific CD8$^+$ T cells measured as in (E–G) after boosting with BNT162b2 or mRNA-1273. Each pie slice represents the mean

number of functions per condition as indicated in the key. Correlations were determined using Spearman’s rank test (A–C, E–G).

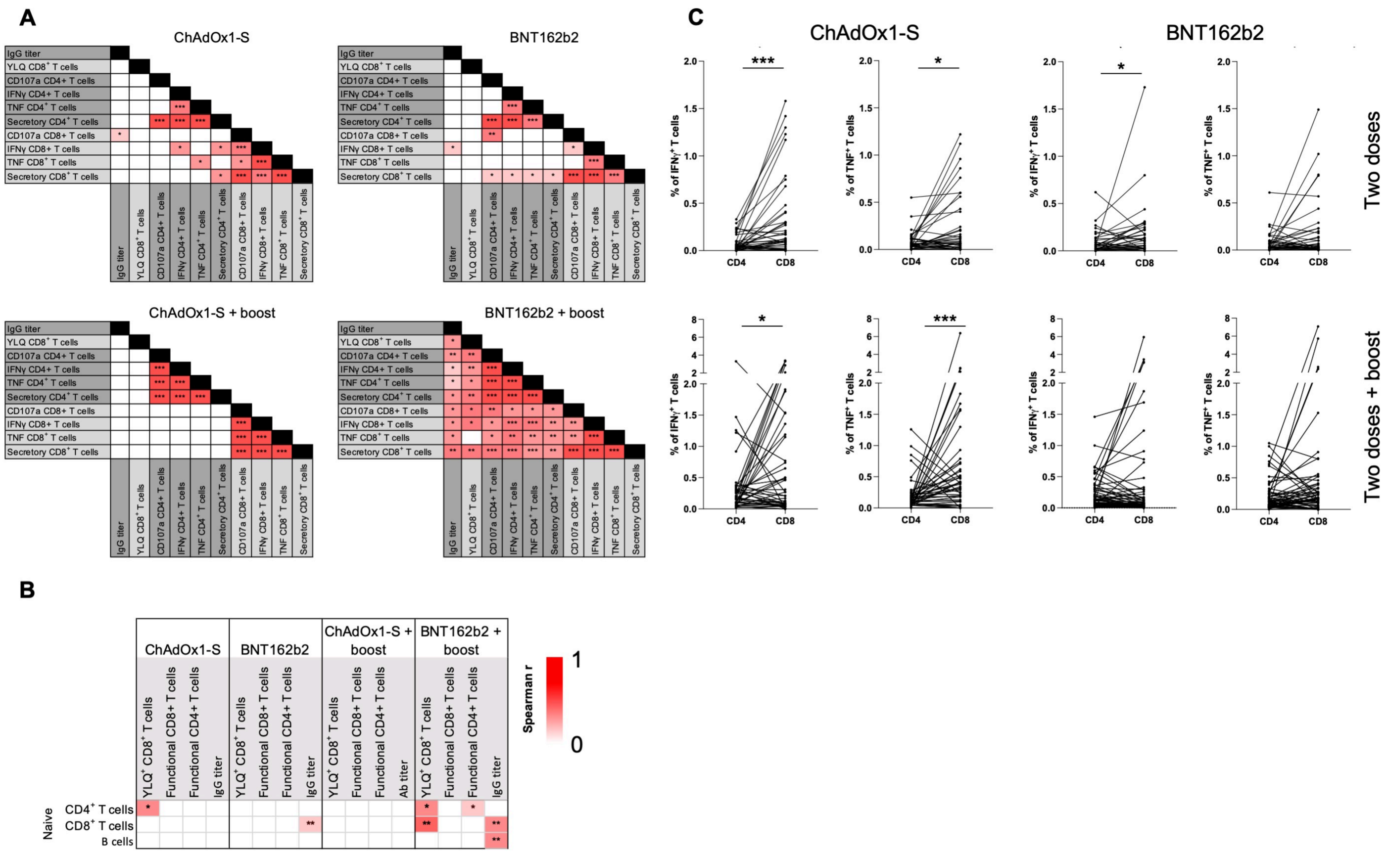


Figure 4

Figure 4

Coordination of cellular and humoral immune responses as a function of immunosenescence after vaccination against SARS-CoV-2. (**A**) Correlations among the indicated measures of adaptive immunity stratified by vaccination schedule. (**B**) Correlations between each measure of adaptive immunity and naive lymphocyte subset frequencies stratified by vaccination schedule. (**C**) Paired differences between spike-specific $CD4^+$ and $CD8^+$ T cell frequencies (ChAdOx1-S, *n* = 42–44; BNT162b2, *n* = 44–45; ChAdOx1-S + boost, *n* = 37; BNT162b2 + boost, *n* = 55–57) measured via the recall induction of IFNγ or TNF after transient expansion stratified by vaccination schedule. Correlations were determined using Spearman’s rank test (A, B). Paired differences were determined using the Wilcoxon matched-pairs signed-rank test (C). *p < 0.05, **p < 0.01, ***p < 0.001.

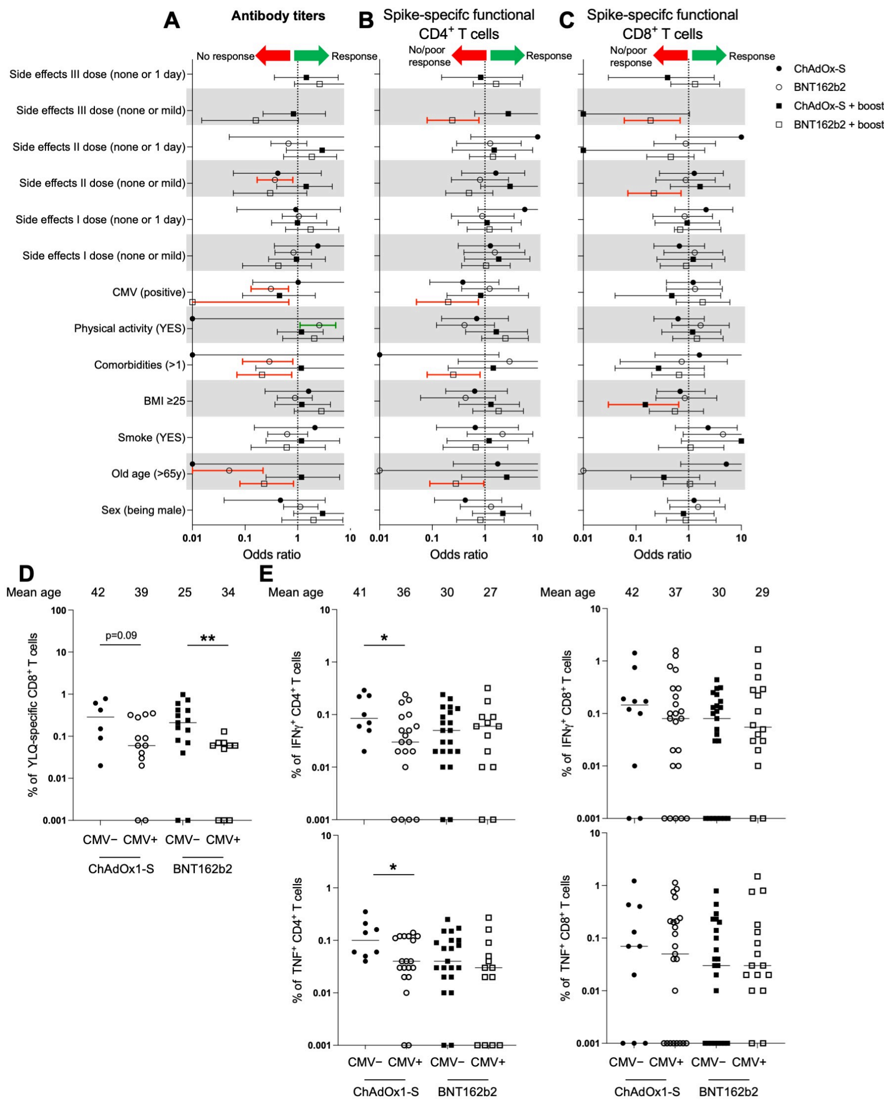
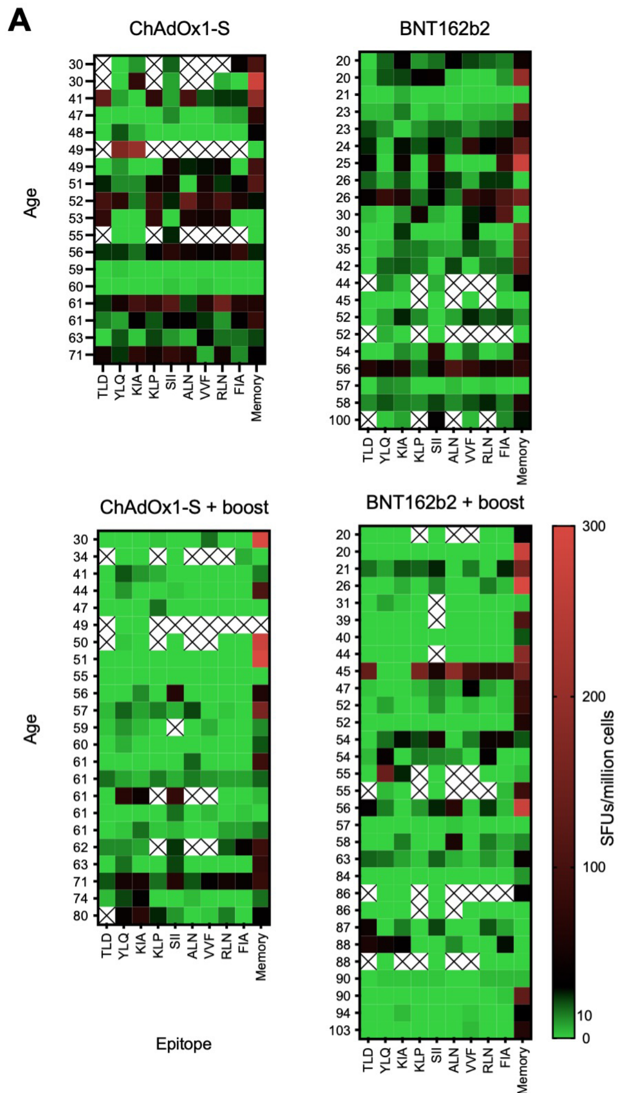


Figure 5

Figure 5

Cellular and humoral immune responses as a function of intrinsic factors after vaccination against SARS-CoV-2. (**A**–**C**) Associations between individual intrinsic factors and RBD-specific IgG titers (A), spike-specific CD4$^+$ T cell frequencies (B), and spike-specific CD8$^+$ T cell frequencies (C) stratified by response status. Colored lines indicate significant odds ratios. (**D**) YLQ-specific CD8$^+$ T cell frequencies measured via tetramer staining after transient expansion from donors aged <50 years stratified as seronegative (ChAdOx1-S, *n* = 6; BNT162b2, *n* = 15) or seropositive for CMV (ChAdOx1-S, *n* = 13; BNT162b2, *n* = 10). (**E**) Spike-specific CD4$^+$ and CD8$^+$ T cell frequencies measured via the recall induction of IFNγ or TNF after transient expansion from donors aged <50 years stratified as seronegative (CD4: ChAdOx1-S, *n* = 8; BNT162b2, *n* = 21; CD8: ChAdOx1-S, *n* = 10; BNT162b2, *n* = 23) or seropositive for CMV (CD4: ChAdOx1-S, *n* = 19; BNT162b2, *n* = 13; CD8: ChAdOx1-S, *n* = 23; BNT162b2, *n* = 16). *p < 0.05, **p < 0.01 (seronegative versus seropositive, Mann-Whitney U test with Bonferroni correction).



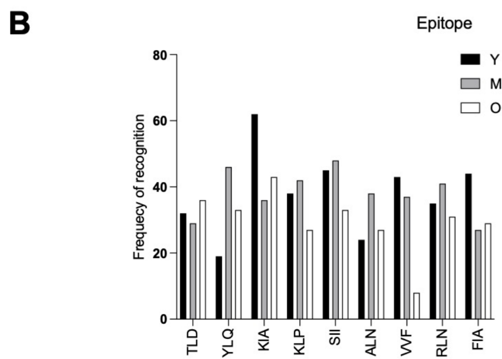


Figure 6

Figure 6

CD8⁺ T cell response diversity as a function of age after vaccination against SARS-CoV-2. (**A**) HLA-A2-restricted spike epitope-specific $CD8^+$ T cell frequencies measured via IFNγ ELISpot assays directly *ex vivo* as a function of age. Data are shown as heatmaps after background subtraction. SFUs, spot-forming units. (**B**) Epitope recognition frequencies stratified by age. All data are shown after background

subtraction. Spike peptides are listed in Supplementary Table S3, and other viral peptides (memory) are listed in Supplementary Table S4.

Supplementary Files

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

- FiguresS1S4.pdf
- Supplementarymaterials.docx