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CD31 + naïve T cells associate with immunosenescence and responsiveness to multiple vaccines in older adults

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Abstract

Background The T cell compartment undergoes significant age-related changes, contributing to the decline of the adaptive immune system and increasing the risk of suboptimal antibody responses to vaccines in older adults. To better understand the association between T cell phenotypes and vaccine responsiveness, we conducted an in-depth analysis of CD4+, CD8+, and γδ + T cells on VITAL cohort participants who are low or high responders to multiple vaccines (influenza, pneumococcal, and SARS-CoV-2).

Results Using spectral cytometry and FlowSOM, we identified detailed phenotypes of naïve, regulatory, and terminally differentiated T cells. We observed that the percentages of CD31 + naïve CD4+, CD31 + naïve CD8+, and CD38 + naïve CD8 + T cells were significantly lower in low vaccine responders. Notably, CD31 + naïve T cell subsets showed a stronger correlation with immune entropy, a measure of cumulative immune system perturbations, than with age itself.

Conclusions These findings suggest that subsets of naïve cells could be associated with weak vaccine responsiveness and immunosenescence. Furthermore, these naive T cell signatures could help predict weak vaccine responses, potentially informing targeted vaccination strategies in older adults.

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Introduction

Aging is associated with a decline in the immune system's ability to respond to pathogens and vaccines, a phenomenon known as immunosenescence [1, 2]. While most components of the immune system change with age, this is particularly apparent in the T cell compartment [3, 4]. These changes include a reduction in naïve T cells and an increase in effector and regulatory T-cell subsets [3, 5, 6].

While age-related alterations in T cells are well-documented, comprehensive insights into T cell phenotypes in individuals who exhibit suboptimal immune responses to vaccines compared to those with robust responses are lacking [4, 7–10]. Since T cells play a key role in

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the adaptive immune system and vaccine responses, a deeper understanding of the T cell compartment is crucial to identify individuals at risk of suboptimal vaccine responsiveness. Our previous analysis of innate, T-cell, and B-cell subsets in the VITAL cohort provided baseline immune profiles [5] but did not include in-depth phenotyping of naïve, memory and terminally differentiated T cell subsets. Given the observed associations between immune phenotype and vaccine responsiveness, we hypothesize that a more detailed investigation of the T cell compartment could enhance these associations [11, 12].

In this study, we compared the in-depth T cell subsets of low and high responders to multiple vaccines (influenza, pneumococcal, and SARS-CoV-2) using samples from VITAL cohort participants [13]. We investigate responsiveness to multiple vaccines as a proxy of immunosenescence, since overall responsiveness to three vaccines provides information about immune functionality of an individual. To study in-depth T cell subsets, we measured an extensive list of markers to gain insights into different conventional αβ + CD4 + and CD8 + T cells, and γδ + T cells. Moreover, we also examined naïve T cells using CD31, indicative of recent thymic emigrants with high proliferative potential and homeostatic maintenance [5, 6, 14, 15]. To better characterize regulatory T cells, we measured markers critical for their suppressive function and stability (Helios, Foxp3, CD25, and CTLA-4) [16, 17]. Additionally, we included markers such as CD28, CD57, and KLRG1 to study terminally differentiated T cells with reduced proliferative capacity, increased cytotoxic potential, and senescence-associated characteristics [18–20]. Finally, we investigated HLA-DR expression in T cells, as previous studies have shown an increase in HLA-DR + T cells in older adults with weaker vaccine responses [21, 22].

Our findings revealed distinct T cell signatures associated with vaccine responsiveness. Low vaccine responders had significantly lower percentages of CD31 + CD4 + naïve, CD31 + CD8 + naïve, and CD38 + CD8 + naïve T cells. These novel immune cell subset differences in low vaccine responders warrant validation in other cohorts and contribute to the discovery of predictors of vaccine responsiveness, aiding the design of targeted vaccination strategies.

Results

Characteristics of the study population

Peripheral blood mononuclear cells (PBMCs) were obtained from 31 individuals aged 25 to 77 from the VITAL cohort based on their vaccine response profiles, age, sex and CMV-seropositivity (Material and Methods) [13].These individuals were categorized into three age groups: young adults (25–49 years, N = 7, median age 29), middle-aged adults (50–64 years, N = 8, median age 60), and older adults (65–77 years, N = 16, median age 72) (Table 1). Sex and CMV-seropositivity were comparable between age groups (Table 1). All participants received a quadrivalent influenza vaccine (QIV) in 2019, the conjugated pneumococcal vaccine (PCV13) in 2020, and two doses of the SARS-CoV-2 mRNA-1273 vaccine in 2021[13].

To study vaccine responsiveness, participants were categorized into two groups: low (N = 17) and high vaccine responders (N = 14) based on their antibody responses to three vaccines (QIV, PCV13, mRNA-1273) (Materials and Methods, Table 1). We calculated pre-vaccination antibody titer-corrected vaccine response quartiles separately for each vaccine and then averaged them per individual. Participants with an average response quartile above or below the median were classified as high or low vaccine responders, respectively. This approach was chosen to provide a more robust measure of overall vaccine responsiveness, as we observed variation in vaccine responses within the same individual [12, 13]. By integrating responses across multiple vaccines, this classification serves as a proxy for immunosenescence a cumulative decline in immune function with aging. Age, sex and CMV seropositivity did not significantly differ between low and high responders ($p > 0.05$, Table 1).

In-depth T cell subsets associated with low or high multiple vaccine response

To better understand age-related changes in T cell subset signatures, we pre-gated three major T cell subsets: CD4+, CD8+, and γδ+ (Supplementary Fig. 1), and analyzed their phenotype using FlowSOM, which allowed us

Table 1 Age, sex, and cytomegalovirus (CMV) seropositivity for age groups and vaccine response groups

Age groups	**median age (range)**	**age *p*-value**	**CMV seropositivity % (*n*)**	**CMV *p*-value**	**male % (*n*)**	**sex *p*-value**	***N***
young adults (YA)	29 (25–48)	YA-MA: 0.011	43 (3)	YA-MA: 0.062	29 (2)	YA-MA: 0.061	7
middle aged adults (MA)	60 (55–62)	MA-OA: 0.002	62 (5)	MA-OA: 0.010	50 (4)	MA-OA: 0.067	8
older adults (OA)	72 (65–77)	OA-YA: <0.0001	62 (10)	OA-YA: 0.065	62 (10)	OA-YA: 0.019	16
all	65 (25–77)		58 (18)		52 (16)		31
Vaccine response groups	**median age (range)**	**age *p*-value**	**CMV seropositivity % (n)**	**CMV *p*-value**	**male % (n)**	**sex *p*-value**	***N***
low responders (LR)	69 (39–76)	LR-HR: 0.068	65 (11)	LR-HR: 0.065	65 (11)	LR-HR: 0.210	17
high responders (HR)	60 (25–77)		50 (7)		36 (5)		14

to identify T cell subsets in an unsupervised manner and study all the markers in our panel simultaneously (Supplementary Figs. 2–5). FlowSOM analysis revealed total of 79 immune subsets of which 29 CD4+, 26 CD8+, and 24 γδ + T cell subsets (Fig. 1a-c, Supplementary Fig. 2). We analyzed FlowSOM identified immune cell subset differences between low and high vaccine responders (Supplementary Table 1) and observed 10 FlowSOM clusters differed significantly(Table 2).

Next, to understand the amount of variation explained by these statistically significant subsets, we used principal component (PC) analysis. PC1 explained 52.9% of the variation, separating low and high vaccine responders along this axis (Fig. 1d). We identified CD31 + naïve (Tn, CD45RA + CCR7 + CD27+) CD4+ (CD4c23) and CD31 + Tn CD8+ (CD8c26) as the most important subsets explaining the variation across PC1 (indicated in red and orange) (Fig. 1e).

The percentage of manually gated CD31 + true naïve (CD95- Tn) T cells within both CD4 + and CD8 + populations (Fig. 1f, g), as well as FlowSOM identified CD31 + naïve CD4 + and CD8 + subsets (Fig. 1h-i) were consistently lower in low responders compared to high responders. Since CD31 + T cell subsets were significantly different between vaccine response groups, we investigated CD31 expression intensity in T cell subsets. Low vaccine responders showed significantly lower CD31 expression intensity in CD8 + T cells (p = 0.010), borderline significantly lower in CD4 + T cells (p = 0.630) and no difference in γδ + T cells (p = 0.720) compared to high responders (Supplementary Fig. 6).

In addition, the percentage of CD4c2 cells with Foxp3 + Helios + CD25hiCTLA-4 + CCR4 + Tem (CD45RA-CCR7-) and heterogeneous HLA-DR expression (HLA-DR+/-) showed a higher percentage in low vaccine responders compared to high responders (Fig. 2j). Conversely, the percentages of CD4c22 (CD4 + Tn Treg: Foxp3 + CD127loCD25hi) (Table 2) and CD8c25 (CD8 + Tn CD38+) subsets (Fig. 2k) were higher in high responders compared to low responders. We investigated whether these Treg differences were present in the overall CD4 + Treg percentages. Therefore, we manually gated CD4 + Tregs (CD127loCD25hi), however, CD4 + Tregs were not statistically different between vaccine response groups (p = 0.450E-01).

Among γδ + T cell subsets, two clusters showed significant differences between low and high responders. CD57 + KLRG1 + CD159a + γδ + Tte (CD45RA + CCR7-CD57 + CD28-) [23] cells (GDc22) were higher, while Helios + CCR4 + γδ + Tcm (CD45RA-CCR7+) cells (GDc11) were lower in low vaccine responders compared to high responders (Fig. 2l, m).

Age group differences among vaccine response associated T cell subsets

Next we analyzed age group differences in FlowSOM-identified immune subsets. Among the 79 total subsets, 20 showed significant differences between age groups after correcting for multiple tests (Table 3). Of these, 6 were also significantly different between vaccine responders (Table 3).

The percentages of CD4c2 (HLA-DR+/- CD4 + Tem Treg) and CD4c22 (CD4 + Tn Treg) subsets differed significantly between both age groups and vaccine responders (Fig. 2a, b; Table 3). Given the unclear role of HLA-DR expression in T cells and its previously reported association with age, we investigated it further. Among CD4 + FlowSOM clusters, the subset with the highest median FOXP3 expression (CD4c2) expressed HLA-DR, indicating these cells are likely CD4 + Tregs. In contrast, HLA-DR + CD8 + T cells (CD8c3, CD8c2) co-expressed CD38 and displayed a more (terminally) differentiated phenotype (CD57+/-CD28-) (Supplementary Fig. 2), suggesting distinct HLA-DR + phenotypes between CD4 + and CD8 + T cells. To further clarify this, we manually gated HLA-DR + T cells across distinct differentiation stages, including naïve (Tn, CD45RA + CCR7 + CD27+), central memory (Tcm, CD45RA-CCR7+), effector memory (Tem, CD45RA-CCR7-) and terminal effector memory T cells re-expressing CD45RA (Temra, CD45RA + CCR7-) cells. HLA-DR + CD8 + T cells comprised up to 35% of Tem and 50% of Temra cells (Supplementary Fig. 7a), while HLA-DR + CD4 + T cells displayed 50% Tem and 40% Tcm phenotypes (Supplementary Fig. 7b). This indicates that HLA-DR + CD8 + T cells are more differentiated than their CD4 + counterparts.

Additionally, the percentage of CD38 + naïve CD8 + T cells (CD8c25) and γδ + CD159 + KLRG1 + Tte (GDc22) which were significantly different between vaccine responders, were significantly lower in older adults compared to younger as well (Fig. 2c, d). Although middle-aged adults showed a higher percentage of CD8c25 and GDc22 compared to older adults, these differences were not statistically significant.

CMV is often found to influence the T cell compartment, especially memory CD8 + T cells, but its association with vaccine responsiveness remains unclear [24–26]. Hence, we investigated whether CMV seropositivity is associated with the above observed vaccine response differing T cell subsets. None of the vaccine response associated T cell subsets were significantly different between CMV + and CMV- individuals (Supplementary Table 2).

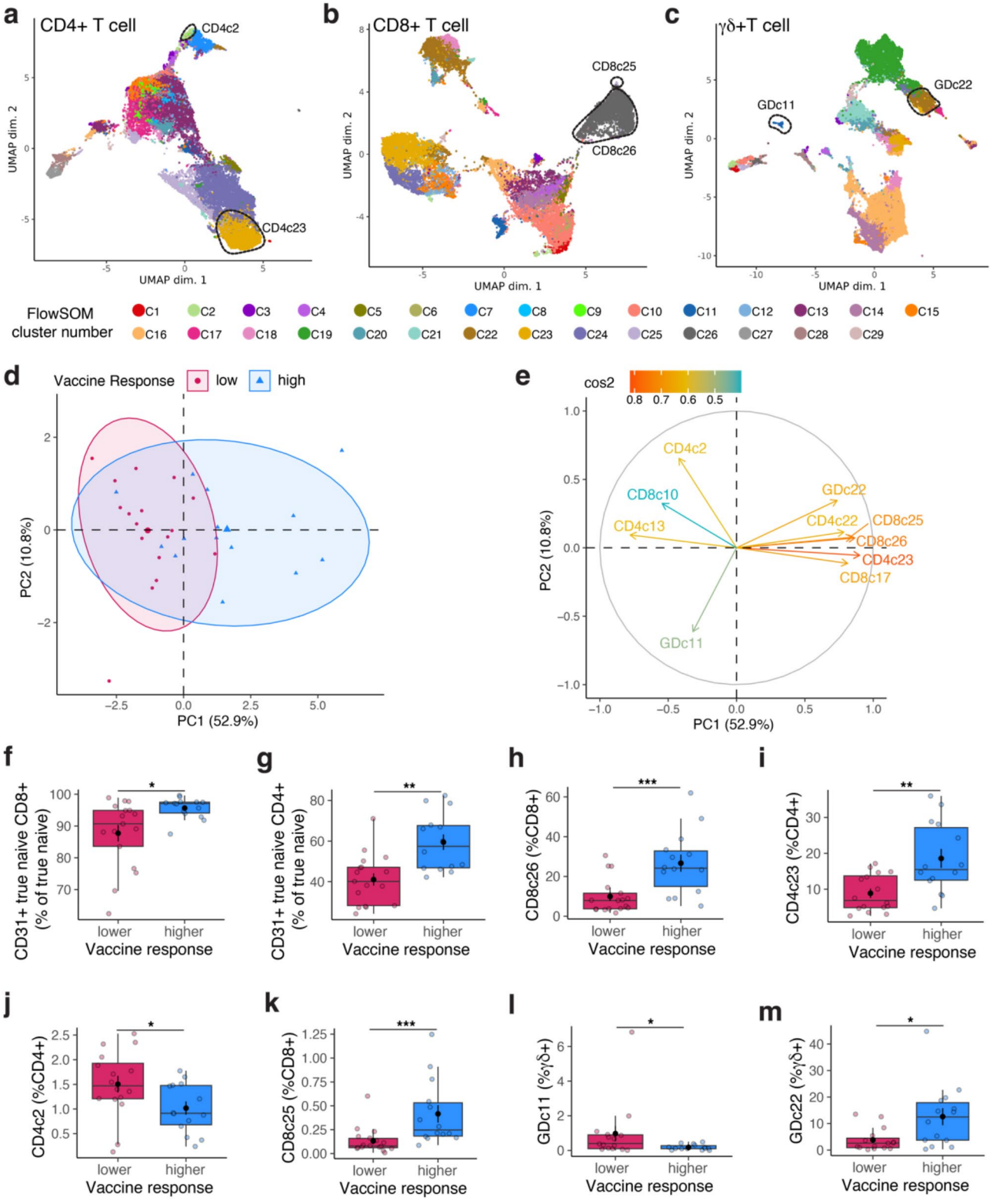

Fig. 1 In-depth T cell subsets associated with low or high multiple vaccine response. (**a**) UMAPs presenting FlowSOM meta clusters for CD4+, (**b**) CD8 + and (**c**) γδ + T cells. (**d**) Projection of the percentage of 10 immune cell subsets that were statistically significant between low and high vaccine responders onto the two Principal Components (PC) (**e**) Contribution of immune cell subsets on PCs colored by their square of cosine (cos2). (**f**) The percentage of manually gated CD31 + true naïve CD8+, (**g**) CD31 + true naïve CD4+, and FlowSOM identified (**h**) CD8c26 (CD31 + naïve CD8 + T cells), (**i**) CD4c23 (CD31 + naïve CD4 + T cells), (**j**) CD4c2 (HLA-DR+/- CD4 + Tem Treg), (**k**) CD8c25 (CD38 + naïve CD8 + T cells), (**l**) GDc11 (Helios + CCR4 + Tcm-like γδ + T cells), (**m**) GDc22 (KLRG1 + CD159a + Tte-like γδ + T cells)in vaccine response groups. CD45RA + CCR7-CD28-CD57+ (Terminal effector, Tte) The significance between vaccine response groups was determined by Mann-Whitney-Wilcoxon test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Table 2 Statistically significant differences in flowsom identified T cell subsets between low and high multiple vaccine responders. Arrows indicate whether the given subset is higher (↑) or lower (↓) in the low vaccine responders compared to high responders

subset	cluster ID	group1	group2	*p*-value	*p*.adj
CD4 + Tem Treg	CD4c2	low responder ↑	high responder	0.042	0.329
CD4 + Tcm	CD4c13	low responder ↑	high responder	0.008	0.104
CD4 + Tn Treg	CD4c22	low responder ↓	high responder	0.000	0.018
CD4 + Tn CD31+	CD4c23	low responder ↓	high responder	0.004	0.071
CD8 + Tem	CD8c10	low responder ↑	high responder	0.032	0.284
CD8 + Helios + CD31+	CD8c17	low responder ↓	high responder	0.004	0.071
CD8 + Tn CD38+	CD8c25	low responder ↓	high responder	0.001	0.019
CD8 + Tn CD31+	CD8c26	low responder ↓	high responder	0.001	0.019
gd + Tcm CCR4 + Helios+	GDc11	low responder ↑	high responder	0.030	0.284
gd + Tte KLRG1 + CD159a+	GDc22	low responder ↓	high responder	0.013	0.144

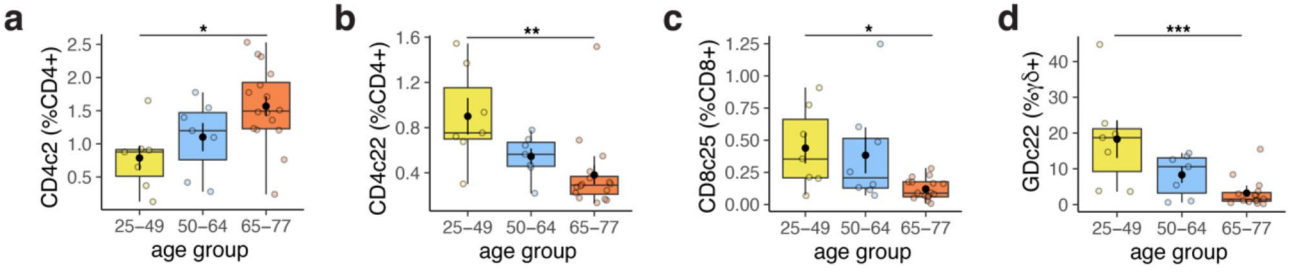

Fig. 2 Age-group differences in immune subsets that are statistically significant between vaccine response groups. (**a**) CD4c2 (HLA-DR+/- CD4 + Tem Treg), (**b**) CD4c22 (CD4 + Tn Treg), (**c**) CD8c25 (CD38 + naïve CD8 + T cells), (**d**) GDc22 (KLRG1 + CD159a + Tte-like γδ + T cells) in age groups. The significance was determined using Kruskal–Wallis tests. Post hoc tests were performed using Dunn's test with the Benjamini-Hochberg method to adjust for multiple comparisons. **p.adj* < 0.05, ***p.adj* < 0.01, ****p.adj* < 0.001

CD31 + naïve T cells show a strong correlation with immune entropy

We have previously described immune entropy, an immune subset network based immunological distance measure that calculates the accumulated immune perturbations with respect to a reference group of young adults with robust vaccine responsiveness. In our previous study, we demonstrated that immune entropy is inversely associated with multiple vaccine responsiveness and serves as a more accurate representation of immunosenescence than chronological age [12]. However, the immune entropy calculation in that study did not include detailed T cell subset information. Here, we investigated how the T cell subsets associated with vaccine response correlate with immune entropy. Given that immune entropy is correlated with age, we also evaluated the strength of the correlations between T cell subsets and age to assess whether these variables are more strongly associated with immune entropy than age.

Of the 10 significantly vaccine response-associated T cell subsets, 9 were significantly correlated with immune entropy (Table 4). Among these, three naïve subsets CD31 + CD4 + T cells (CD4c23, r=-0.54, p = 0.003), CD31 + CD8 + T cells (CD8c26, r=-0.61, p < 0.0013.14E-04), and CD38 + CD8 + T cells (CD8c25, r=-0.72, p < 0.001) demonstrated stronger and more significant correlations with immune entropy than with age (CD4c23, r=-0.3, p = 0.111; CD8c26, r=-0.43, p = 0.016; CD8c25, r=-0.58, p < 0.001) (Fig. 3a-c). Similarly, CD4 + Tcm (CD4c13, r = 0.56, p = 0.002) and CD8 + Tem (CD8c10, r = 0.52, p = 0.003) also showed stronger correlations with immune entropy compared to age. These findings suggest that CD31 expression in naïve T cells may offer additional insights for distinguishing low and high vaccine responders, particularly in older adults.

Discussion

The T cell compartment in the human immune system undergoes profound changes throughout life, with a notable decline in adaptive immune responses in later life, often associated with suboptimal immune responses to vaccines [3, 7, 11]. We identified CD31 + naïve CD4+, CD31 + naïve CD8 + and CD38 + naïve CD8 + T cells were significantly lower in low vaccine responders compared to high responders. Additionally, these subsets showed stronger correlations with immune entropy than with chronological age.

The naïve T cell compartment undergoes a significant decline with age [5, 6]. Beyond a mere reduction in the percentage and number of naïve T cells, nuanced differences in immune phenotypes are essential to understanding the state of the naïve T cell compartment in the context of aging and immunosenescence. Since the percentage of CD31 + naïve CD4 + and CD8 + T cells were

Table 3 Statistically significant differences in flowsom identified T cell subsets between age groups divided into two sections based on being vaccine response associated or not. Arrows indicate whether the given subset is higher (↑) or lower (↓) in the group1 compared to group2

Immune subsets significantly different between age groups and vaccine response groups					
subset	**cluster ID**	**group1**	**group2**	***p*-value**	***p*.adj**
CD4 + Tem Treg	CD4c2	YA↓	OA	0.006	0.019
CD4 + Tn Treg	CD4c22	YA↑	OA	0.001	0.004
CD8 + Tem	CD8c10	YA↓	MA	0.005	0.007
CD8 + Tem	CD8c10	YA↓	OA	0.005	0.007
CD8 + Helios + CD31+	CD8c17	YA↑	OA	0.001	0.003
CD8 + Tn CD38+	CD8c25	YA↑	OA	0.008	0.023
gd + Tte KLRG1 + CD159a+	GDc22	YA↑	OA	0.001	0.003
Immune subsets significantly different between age groups but not vaccine response groups					
subset	**cluster ID**	**group1**	**group2**	***p*-value**	**p. adj**
CD4 + Tem Treg-like	CD4c6	YA↓	OA	0.006	0.018
CD4 + Tem Treg-like	CD4c7	YA↓	OA	0.015	0.044
CD4 + Tn CD31-	CD4c24	YA↓	MA	0.008	0.011
CD4 + Tn CD31-	CD4c24	YA↓	OA	0.001	0.002
CD4 + Tte	CD4c27	YA↑	MA	0.016	0.024
CD4 + Tte	CD4c27	MA↓	OA	0.005	0.015
CD8 + ICOS + CCR4+	CD8c1	YA↓	OA	0.006	0.018
CD8 + Tcm	CD8c9	YA↓	MA	0.031	0.046
CD8 + Tcm	CD8c9	YA↓	OA	0.001	0.004
gd + Treg-like	GDc1	YA↓	OA	0.003	0.008
gd + Treg-like	GDc1	MA↓	OA	0.030	0.045
gd + CD4 + Helios+	GDc2	YA↓	OA	0.006	0.018
gd + CD4 + Helios+	GDc2	MA↓	OA	0.021	0.032
gd + CD4 + CD38 + ICOS + CTLA-4+	GDc3	YA↓	OA	0.001	0.002
gd + CD4 + Tcm CXCR3 + CCR6+	GDc4	YA↓	OA	0.003	0.009
gd + CD4 + CD95 + CCR4+	GDc10	YA↓	OA	0.008	0.024
gd+ CD159a + CX3CR1+	GDc17	YA↓	OA	0.018	0.026
gd+ CD159a + CX3CR1+	GDc17	MA↑	OA	0.002	0.005
gd+ CD45RA + KLRG1+	GDc20	MA↑	OA	0.006	0.019
gd+ KLRG1+	GDc21	YA↓	MA	0.024	0.045
gd+ KLRG1+	GDc21	MA↑	OA	0.030	0.045

Young adults (YA), Middle-aged adults (MA), Older adults (OA)

Tn (naive): CD45RA + CCR7 + CD27+, Tem (effector memory): CD45RA-CCR7-, Tcm (central memory): CD45RA-CCR7+

Treg (regulatory): CD25 + + Foxp3+, Tte (terminal effector): CD45RA + CCR7-CD28-CD57+

Table 4 Correlations between vaccine response associated T cell subsets, age and immune entropy

variable 1	**variable 2**	**cor (rho)**	***p*-value**	***p*.adj**
immune entropy	age	0.5	0.004	0.007
CD4c2	immune entropy	0.25	0.185	0.185
CD4c2	age	0.51	0.004	0.007
CD4c13	immune entropy	0.56	0.001	0.003
CD4c13	age	0.35	0.059	0.069
CD4c22	immune entropy	-0.57	0.001	0.003
CD4c22	age	-0.66	0.000	0.001
CD4c23	immune entropy	-0.54	0.002	0.005
CD4c23	age	-0.3	0.111	0.117
CD8c10	immune entropy	0.52	0.003	0.006
CD8c10	age	0.31	0.089	0.099
CD8c17	immune entropy	-0.61	0.000	0.001
CD8c17	age	-0.71	0.000	0.000
CD8c25	immune entropy	-0.72	0.000	0.000
CD8c25	age	-0.58	0.001	0.002
CD8c26	immune entropy	-0.61	0.000	0.001
CD8c26	age	-0.43	0.016	0.020
GDc11	immune entropy	0.5	0.006	0.009
GDc11	age	0.48	0.009	0.012
GDc22	immune entropy	-0.46	0.012	0.016
GDc22	age	-0.66	0.000	0.001

significantly higher in high vaccine responders compared to low responders, this suggests that CD31 could be a relevant variable in the context of vaccine responsiveness. CD31 + naïve T cells, being less differentiated and having higher proliferative potential, may respond more effectively to vaccine antigens by generating robust and sustained immune responses [27]. Therefore, the higher expression of CD31 and higher percentages of CD31 + naïve T cells in high responders might indicate a more youthful and functionally competent T cell pool, capable of mounting stronger and more effective immune responses upon vaccination. In contrast, a diminished CD31 expression and CD31 + subsets could be a signature of weak vaccine responsiveness and immunosenescence. Another notable observation in naïve T cells was the higher percentage of CD38 + naïve CD8 + T cells in high vaccine responders. CD38 has been reported as an important marker for naïve T cells, crucial for modulating their metabolic activity and maintaining these cells in a quiescent state and ensuring readiness for rapid response upon activation [28]. Higher percentages of CD38 + CD8 + naïve T cells therefore may contribute to the more robust vaccine responsiveness observed in high responders.

Previously, we developed a measure called immune entropy to quantify immune perturbations in the immune subset network. In short, immune entropy takes into total immune subset network into account to calculate immunological distance from a reference group of young adults who showed robust multiple vaccine

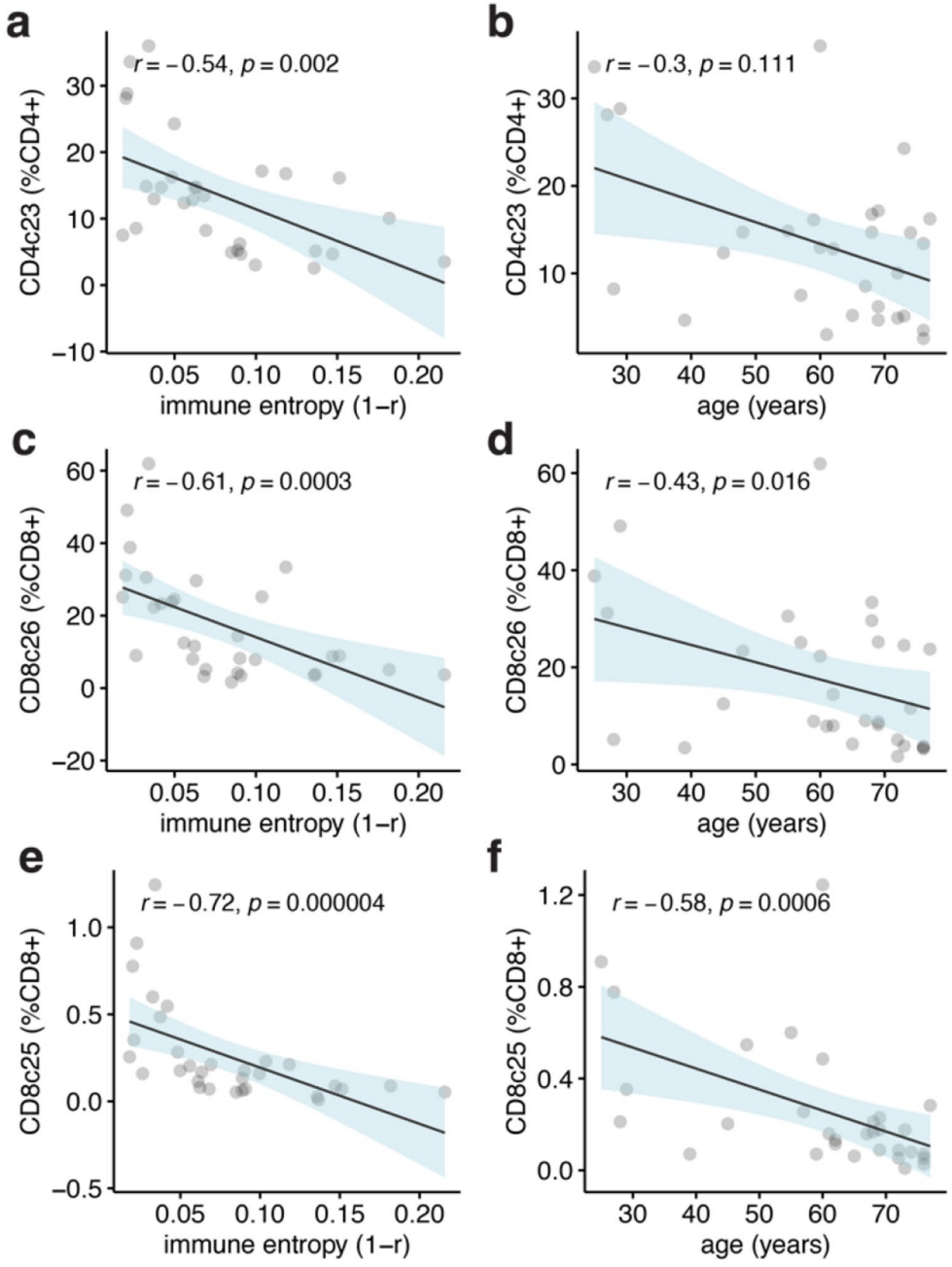

Fig. 3 CD31 and CD38 expression in naïve T cell subsets show a stronger correlation with immune entropy than age. (**a**) Spearman correlation between the percentage of CD31 + naïve CD4 + T cells (CD4c23) and immune entropy, and (**b**) age. (**c**) Spearman correlation between the percentage of CD31 + naïve CD8 + T cells (CD8c26) and immune entropy, and (**d**) age. (**e**) Spearman correlation between the percentage of CD38 + naïve CD8 + T cells (CD8c25) and immune entropy, and (**f**) age. Spearman's rho (r)

responsiveness. We have previously shown that immune entropy reflect immune aging better than chronological age and are significantly associated with responsiveness to multiple vaccines even after age and sex corretion [12]. Since CD31 + naïve T cell subsets were more strongly and inversely associated with immune entropy compared to age, and significantly different in low and high vaccine responders, this suggests that CD31 expression in naïve T cells could be associated with immune aging and immunosenescence. Furthermore, these findings highlight that incorporating CD31 measurement into the immune entropy model could refine this biomarker, leading to more accurate predictions of vaccine responsiveness and enabling targeted interventions to enhance immune function in older populations.

HLA-DR expression increases with age across CD4 + and CD8 + T cells but the phenotype and functionality of HLA-DR + T cells and their relevance to immune aging remains poorly understood. In our study, we observed that HLA-DR + CD4 + T cells exhibited a regulatory T cell-like phenotype, expressing Foxp3 and CTLA-4. HLA-DR + CD4 + Tregs have higher regulatory cell function than HLA-DR- CD4 + Tregs [29]. The marked increase in HLA-DR+/-Foxp3 + CTLA4 + CD4 + T cells in older age groups aligns with the reported expansion in the regulatory T cell compartment with age [30]. The percentage of FlowSOM-identified HLA-DR+/- CD4 + Tregs was more prevalent among older adults and low vaccine responders, while manually gated total CD4 + Tregs were not significantly different. This suggests that nuanced differences among CD4 + Treg subsets compared to the percentage of total CD4 + Treg (CD127loCD25hi) could be more relevant for immune aging and vaccine responsiveness. On the other hand, the potential function of HLA-DR + CD8 + T cell remains elusive. Our in-depth phenotyping suggested that HLA-DR + CD8 + T cells are a subset of highly differentiated (CD45RA + CCR7-CD57 + CD28-) T cells in older adults. Moreover, HLA-DR + CD8 + T cell clusters showed CD38 expression as well. Higher percentages of HLA-DR + CD38 + CD8 + T cells were observed in viral infections such as flu, HIV-1 and SARS-CoV-2 and immune regulatory disorders, therefore could be related with chronic infection or an activated state [31–33]. Further functional characterization of these cells could help us understand their role in shaping the immune network, potentially involving pathways related to immune modulation.

In contrast to αβ + T cells, the functional properties and phenotypes of γδ + T cells are less understood, but their relevance in immune aging and vaccine responses is becoming increasingly evident [34–37]. We identified two γδ + T cell subsets (GDc11 and GDc22) that differed between low and high vaccine responders, though their specific functions remain unclear. GDc11 exhibited Helios + CCR4 + Tcm-like phenotype. Helios, although typically a regulatory CD4 + T cell marker, does not correlate with suppressive function in γδ + T cells [38, 39]. The expression of CCR4 in γδ + T cells could indicate roles in Th1 phenotype localization during infections and inflammation, or involvement in skin-homing and self-renewal capabilities [40, 41]. Given the rarity of this subset, GDc11 might represent regulatory-like tissue-resident T cells circulating in the blood. GDc22 exhibited CD159a (NKG2A) expression, which is associated with high cytotoxic potential in γδ + T cells [42]. CD57 + KLRG1 + expression in GDc22 cluster resembles a highly differentiated CD8 + T cell phenotype [18, 43, 44]. Nevertheless, we cannot explain the possible roles of these subsets based on marker expressions alone. Further studies are needed to investigate how they interact with other immune cells and how they are modulated during aging and vaccination.

Our study has certain limitations. Due to the small sample size, our findings may not be generalizable to a larger population. CMV significantly impacts the T cell pool, complicating the use of T cell variables as predictive markers of vaccine responsiveness. Ideally, identifying immune variables that are unaffected by CMV-seropositivity but still correlate with vaccine responsiveness would be optimal. Although we matched groups for CMV-seropositivity and did not observe differences in CD31 + naïve T cells related to CMV status, these effects may have been missed due to our small sample size. Even though the baseline state of the T cell pool and the role of T cells in vaccine responses and immune aging are key players, here we do not investigate B cells which are another key subset in antibody response to vaccines. While we used well-characterized samples based on vaccine responsiveness, the functional properties of identified immune subsets, such as Helios + CCR4 + γδ + T cells, remain unknown and require further experiments. Future studies should include larger sample sizes, longitudinal designs, and functional assays to validate and extend our findings.

Conclusions

These findings provide deeper insights into the T cell phenotypes associated with immune aging and vaccine responsiveness. Our findings highlight the significance of CD31 + naïve T cells and their association with immune entropy, suggesting that these immune subsets could be of an interest to improve identification of individuals at risk of immunosenescence. Understanding the mechanisms behind these phenotypic changes could lead to improved strategies for boosting immune function and vaccine efficacy. Ultimately, this could translate into more effective vaccination programs and personalized interventions to enhance immune protection in older adults.

Materials and methods

Cohort description

This study used samples from the longitudinal intervention study VITAL reported in detail elsewhere [5, 13]. In short, individuals 25 to 98 years old were recruited. Individuals were excluded when they use or used immune-modulatory drugs or have a disease that make them immunocompromised, including recipient of an organ- or bone marrow transplant, used high-dose of daily corticosteroids or received chemotherapy in the last 3 years. Detailed inclusion and exclusion criteria have been reported previously [13].

From the VITAL cohort, we selected 31 individuals who are sex and CMV seropositivity matched for three

age groups: young adults (25–49 years old), middle-aged adults (50–64 years old), and older adults (65–77 years old) who were characterized as low or high responders to multiple vaccines (section Serum antibodies and vaccine response profiles). Participants were further categorized as low or high responders to multiple vaccines (section Serum Antibodies and Vaccine Response Profiles). The selection of low and hig responders was designed to minimize the influence of age, sex, and CMV seropositivity ($p > 0.05$), thereby reducing potential confounding effects on vaccine response and immunosenescence. This approach was based on previous findings from the VITAL cohort, where older male participants were enriched among low multiple vaccine responders [13].

Sample collection and Preparation

PBMCs were isolated from whole blood samples at the start of the VITAL study, before influenza vaccination, using density gradient centrifugation with Lymphoprep (Progen). The cells were resuspended in a cryoprotective solution containing 20% Fetal calf serum (FCS, Hyclone, GE Healthcare, Chicago, Illinois, USA) and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich). The PBMCs were aliquoted into cryovials, gradually frozen and stored at -135 °C until further analysis. Cryopreserved PBMC samples were rapidly thawed in a 37 °C water bath. The cells were immediately diluted in RPMI 1640 medium (Gibco) supplemented with 10% FCS, and centrifuged at 300×g for 10 min, and resuspended in fresh medium. Cell viability and counts are determined using Beckman Coulter z2 (Beckman Coulter).

Immune phenotyping

PBMCs were stained using a panel of fluorochrome-conjugated anti-human antibodies. For surface antigens CCR4 BV605 (clone: L281H4, supplier: BD Biosciences), CCR6 BV711 (G034E3, Biolegend), CCR7 BV421 (G043H7, Biolegend), CD127 APC-R700 (HIL-7R-M21, BD Biosciences), CD14 Superbright 436 (61D3, ThermoFisher), CD159a APC (REA110, Miltenyi Biotech),, CD19 Superbright 436 (HIB19, ThermoFisher), CD25 cFluorBYG710 (BC96, Cytek), CD27 APC-H7 (M-T271, BD Biosciences), CD28 BV650 (CD28.2, Biolegend), CD3 BUV805 (SK7, BD Biosciences), CD31 BUV563 (L133.1, BD Biosciences), CD38 APC/Fire 810 (HIT2, Biolegend), CD4 cFluorYG584 (SK3, Cytek), CD45RA BUV395 (5H9, BD Biosciences), CD56 Superbright 436 (TULY56, ThermoFisher), CD57 BB515 (NK-1, BD Biosciences), CD8 cFluor V547 (SK1, Cytek), CD95 PE-Cy5 (DX2, Biolegend), CX3CR1 BUV737 (2A9-1, BD Biosciences), CXCR3 PE-Cy7 (G025H7, Biolegend), CXCR5 BV750 (RF8B2, BD Biosciences), HLA-DR BV570 (L243, Biolegend), ICOS BUV661 (DX29, BD Biosciences), KIR2D PE (NKVFS1, Miltenyi Biotech), KIR3DL1/DL2 PE (5.133, Miltenyi Biotech), KRLG1 VioBlue (REA261, Miltenyi Biotech), PD-1 BV785 (EH12.2H7, Biolegend), TCR γδ PerCP-Vio 700 (REA591, Miltenyi Biotech), TIGIT BV480 (741182, BD Biosciences), for intracellular/intranuclear antigens, CTLA-4 PE-CF594 (BNI3, BD Biosciences), Foxp3 BB700 (236 A/E7, BD Biosciences), Helios Alexa Fluor 647 (22F6, Biolegend) were used. Cell viability dye Live Dead Blue (ThermoFisher) was used to stain dead cells. In the surface antigen staining mix True-Stain Monocyte Blocker (Biolegend) was added and all antibody mixes were prepared in Brilliant Stain Buffer Plus (BD Biosciences). All staining steps were performed in the dark at room temperature. For the intracellular/intranuclear antigen stainings, cells were fixed Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) following the manufacturer's instructions. During analysis, we observed a suboptimal stain index for CXCR5, which may have affected its resolution. However, some CXCR5hi populations were still detected in FlowSOM heatmaps and UMAP projections. Therefore, interpretations of CXCR5 + subsets should be made with caution.

All samples were acquired Cytek Aurora 5 L (Cytek Biosciences) and unmixed using SpectroFlo (v3.1.0). The cytometer was calibrated using manufacturer-supplied calibration beads to ensure accurate fluorescence intensity measurements. Technical control PBMC samples from the same individual were taken along for each experiment to track cytometry performance and potential batch effect issues.

Spectral cytometry data analysis

Unmixed samples were manually gated in FlowJo (V10.7.1, Tree Star) (Supplementary Fig. 1). CD14, CD19 and CD56 are used as dump channels to gate out monocytes, B cells and Natural killer cells. KIR2D and KIR3DL1/DL2 antibodies are used together to define panKIR population. Furthermore, pre-gated (single cells, lymphocytes, dump-, live, CD3+) samples were exported as three major T cell subsets: CD4 + CD8-γδ- (CD4 + T cells), CD8 + CD4-γδ- (CD8 + T cells) and γδ + T cells, which were imported into R for unsupervised analysis utilizing the RADIANT pipeline. In short, samples were cleaned using PeacoQC [45] and archsinh transformed using optimized cofactors for each channel per major T cell subset. Batch effects were not detected, which was assessed by comparing marker expression profiles of technical controls in histograms and clustering of samples and technical controls on Multidimensional scaling plots (Supplementary Fig. 8a-c). Furthermore, samples were clustered using FlowSOM [46], and frequencies of meta clusters and MFI of antigens were exported to R for downstream analysis.

Immune entropy

Immune entropy was calculated using whole blood immune subsets collected at the pre-vaccination timepoint before the administration of the first vaccine as described previously [12]. In short, immune entropy quantifies the degree of deviation in an individual's immune profile from a reference group of younger individuals, serving as a measure of immune system perturbation and dysregulation. This is determined by computing the correlation distance (1 - Spearman's rho) between an individual's immune cell composition and the median immune cell composition of the reference group. The calculation incorporates a total of 59 immune cell subset variables, ensuring that it captures the overall immune network structure rather than focusing on individual cell types. Immune entropy is a candidate functional biomarker of immune aging, reflecting cumulative changes in the immune system that are not fully explained by chronological age. A higher immune entropy score indicates a greater degree of immune dysregulation, which has been linked to weaker vaccine responsiveness. Since immune entropy associates with vaccine responsiveness after correcting for age and sex effect, in this study immune entropy has been used to assess the relevance of in-depth T cell subsets in terms of immunosenescence.

Cytomegalovirus seropositivity

Immunoglobulin G antibodies against CMV were quantified in serum collected before vaccination by a multiplex immunoassay developed in-house [47]. Seropositivity thresholds were adapted from a previous study [48]. For CMV, a concentration of < 7.5 relative units (RU) ml $^{-1}$ was categorized as seronegative and ≥ 7.5 RU ml $^{-1}$ as seropositive.

Serum antibodies and vaccine response profiles

Serum antibody measurements for the VITAL and VITAL-Corona studies have been described in detail elsewhere [13]. Briefly, antibody levels were measured pre-vaccination and 28 days post-vaccination for each vaccine: H3N2 hemagglutination inhibition titer for QIV, IgG concentrations against the 13 pneumococcal serotypes for PCV13, and IgG binding antibody units (BAU) against the SARS-CoV-2 Spike S1 protein for mRNA-1273. Pre-vaccination antibody level adjusted triple vaccine response quartiles were calculated as previously described [12]. Due to the variation across vaccine responses within the same individual and to get a better proxy for immunosenescence, triple vaccine responsiveness has been used instead of per vaccine response. For this study, response quartiles lower than the median triple vaccine response quartiles (TQ1 and TQ2) were combined to define the "low" response category, while response quartiles higher than the median triple vaccine response quartile (TQ3 and TQ4) were combined to define the "high" response category.

Statistical analyses

Data handling, statistical analyses, and visualization were performed in R (version 4.2.2) and R Studio (version 2022.12.0.353).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12979-025-00504-0.

Supplementary Material 1

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Author contributions

AC performed the data acquisition. AC, MV performed methodology. AC visualized the data. AC wrote the original draft. EB was responsible for data management. JvB was involved in sample selection AC, MvdH, DvB, AB are involved in writing and editing. All authors critically revised the manuscript before publication.

Data availability

The datasets containing participant-specific data used in the current studies are available under restricted access to comply with EU legislation on the General Data Protection Regulation (GDPR) and participant privacy and ethical rights, but are available from the corresponding author on reasonable request. The codes used in the manuscript are available from GitHub. https://github.com/alpercevirgel/Aurora_indepth_Tcellhttps://github.com/alpercevirgel/RADIANT.

Declarations

Ethics approval and consent to participate

Ethical approval for the study was granted by the Medical Research Ethics Committee Utrecht.

Consent for publication

All participants provided written informed consent, and all procedures were conducted in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

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