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AN EXPLORATIVE BIOMARKER STUDY FOR VACCINE RESPONSIVENESS AFTER A PRIMARY MENINGOCOCCAL VACCINATION IN MIDDLE-AGED ADULTS

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Abstract

Prevention of infectious diseases in the elderly is essential to establish healthy ageing. Yet, immunological ageing impairs successful vaccination of the elderly. Predictive biomarkers for vaccine responsiveness in middle-aged adults may help to identify responders and non-responders before reaching old age. Therefore, we aimed to determine biomarkers associated with low and high responsiveness towards a primary vaccination in middle-aged adults, for which a tetravalent meningococcal vaccine was used as a model.

Middle-aged adults (50-65 years of age, N=100), receiving a tetravalent meningococcal vaccination, were divided into low and high responders using the functional antibody titers at 28 days post-vaccination. A total of 48 parameters, including absolute numbers of immune cells and serum levels of cytokines and biochemical markers, were determined pre-vaccination in all participants. Heat maps and multivariate redundancy analysis (RDA) were used to reveal immune phenotype characteristics and associations of the low and high responders.

Several significant differences in pre-vaccination immune markers were observed between the low and high vaccine responders. Moreover, RDA analysis revealed a significant association between the pre-vaccination immune phenotype and vaccine responsiveness. In particular, our analysis pointed at high numbers of CD4 T-cells, especially naïve CD4 and Treg subsets, to be associated with low vaccine responsiveness. In addition, low responders showed lower pre-vaccination IL-1Ra levels than high responders.

This explorative biomarker study shows associations between the pre-vaccination immune phenotype and vaccine responsiveness after a primary meningococcal vaccination in middle-aged adults. Consequently, these results provide a basis for predictive biomarker discovery for vaccine responsiveness that will require validation in larger cohort studies.

Introduction

Prevention of infectious diseases in the elderly is essential to establish healthy ageing in the rapidly growing ageing population. Yet, immunological ageing impairs successful vaccination in the elderly [1-3]. Timely vaccination of middle-aged adults may be an alternative option to strengthen the memory immunity before reaching old age. Previously, we showed that a primary meningococcal vaccination, containing antigens towards which no or very low pre-vaccination immunity exists, was highly immunogenic in middle-aged adults [4]. Current research focusses on the identification of immune markers in older individuals to be able to predict the vaccine responders and non-responders [5, 6]. At present, the discovery of these predictive immune markers at advanced age is challenging and results are not unambiguous.

Potential biomarkers for vaccine responsiveness may relate to shifts in the immune phenotype from naïve to more memory cells during ageing. This phenomenon occurs especially in the T-cell compartment and is caused by thymus involution [7-12]. Accordingly, the responsiveness to a yellow fever vaccine was found positively associated with the numbers of circulating naïve CD4 T-cells that had recently left the thymus [13]. Infection with persistent viruses, such as cytomegalovirus (CMV), enhances the numbers of late-differentiated T-cells and consequently may accelerate immunological ageing [14, 15]. High numbers of these late-differentiated T-cells were negatively associated with influenza and varicella zoster (VZV) vaccine responses [16, 17]. In addition, increased numbers of regulatory T-cells (Tregs) are observed at old age [18, 19] which may underlie the lower responsiveness to the influenza and VZV vaccinations [17, 20].

Age-associated changes in the B-cell compartment have also been reported and include a decrease in naïve B-cells and a subsequent increase in late-differentiated and exhausted B-cells, as well as B-cells with inflammatory characteristics [21-23]. Several vaccination studies described a positive correlation between the frequencies of pre-vaccination Ig switched memory B-cells and the responsiveness to influenza and hepatitis B vaccines [16, 22, 24-26], whereas, late/exhausted (CD27-IgD-) memory B-cells were negatively correlated with the response to the influenza vaccine [22]. Moreover, B-cell expression levels of activation-induced cytidine deaminase (AID) and TFN- α after *in vitro* stimulation were found predictive for humoral responses after influenza vaccination [22, 25, 27-29].

In addition, several innate immune functions, gene signatures, or miRNA expressions were associated with influenza vaccine responsiveness [24, 30, 31]. Moreover, the age associated increase in inflammatory mediators, also known as 'inflammageing' [32-35], as well as modified expression of biochemical markers, such as dehydroepiandrosterone sulphate (DHEAs) [36] and vitamin D [37], might affect the immune function at advancing age [38]. Also, a range of vaccination responses, for example to diphtheria, tetanus and influenza, are substantially influenced by vaccine specific pre-vaccination immunity [26, 30].

The studies mentioned provide some promising predictive biomarkers that require validation in other cohort studies. Correspondingly, in this study we aimed to explore differences in the pre-vaccination immune phenotype between low and high vaccine responders towards a primary immune response upon a meningococcal vaccination in middle aged adults.

Methods

Study design

Data from 100 middle-aged (50-65 years of age, 50% males) adults who received the tetravalent meningococcal vaccine conjugated to tetanus toxoid was used in this explorative biomarker study. These participants were included in a larger cohort study, of which exclusion criteria and study procedures are described elsewhere [4]. In short, pre-vaccination blood samples were drawn from all participants as well as 28 days, and 1 year post-vaccination blood samples. Serum samples were collected at the different time points using serum clotting tubes (BD Biosciences) and were immediately kept cold and stored within 4 hours in aliquots at -20°C and -80°C before further use. Blood samples were collected in tubes containing lithium heparin (BD Biosciences) for detailed cellular immune phenotyping prior to vaccination. Subsequently, different immune parameters, i.e. absolute immune cell counts, serum cytokines, CMV specific antibodies, and biochemical markers were measured in the pre-vaccination blood samples of these participants. Meningococcal specific functional antibody titers were measured in the pre-vaccination, as well as 28 days and 1 year post-vaccination samples. A schematic overview of the study outline is depicted in **Figure 1**. In addition, all participants filled in a short health questionnaire.

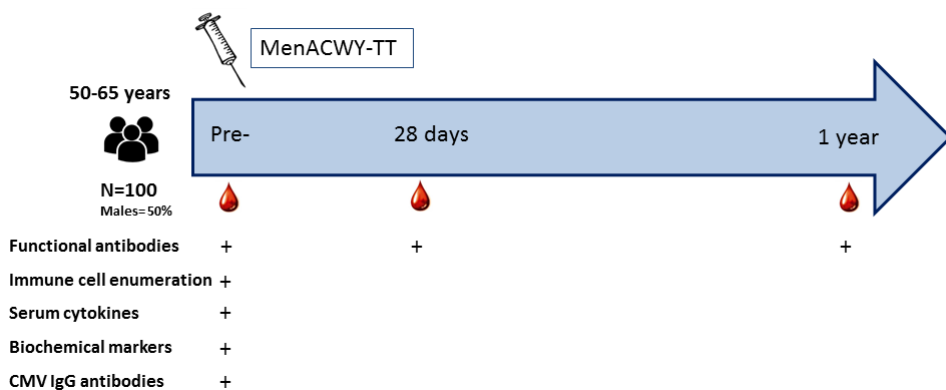


Figure 1. Study outline

Participant selection

Functional antibody titers for the 3 different meningococcal groups (Meningococci C, W, and Y) were measured with the serum bactericidal antibody assay in 100 middle-aged adults, as previously described [4, 39, 40]. Meningococci A specific analysis was left out, due to interference of cross-reactive antibodies in the antibody assays. A functional antibody titer of 8 was considered to be protective, whereas a functional antibody titer of 128 was applied as a more conservative long-term correlate of protection [4, 39].

The quartiles of the functional antibody titers 28 days post-vaccination were calculated. Participants with a functional antibody titer matching the corresponding titer of the first quartile or below were considered low responders, whereas those matching the titer of the 3rd quartile or above were considered high responders. Since part of the participants showed antibody titers equal to the cut-off value, the lowest and highest quartile do not include 25 % of the participants. In total, 25, 46, and 40 low responders and 27, 35, and 34 high responders were defined for MenC, MenW, and MenY, respectively.

Flow cytometric analysis

At the pre-vaccination time point the absolute numbers of a broad range of immune cell subsets were determined as described previously [41-43]. In brief, the absolute numbers of lymphocytes, T-cells, B-cells, NK-cells, monocytes, and granulocytes were measured in fresh whole blood samples (within 18 hours after collection) using TruCOUNT tubes. Gating strategies as well as a detailed description of the antibodies used were as published previously [41]. An overview of the phenotype definitions of the different cellular subsets measured is depicted in **S. Table 1**. These absolute cell numbers were also used to calculate the ratios between the (memory) Treg cells and the CD4+CD45RO⁺ effector memory T (TemRO) cells as well as between the CD4 naïve or CD4+CD45RA+CD25^{dim} cells and the CD4 memory cells. The CD4 memory cells were defined as the sum of the CD4 central memory (CM), CD4 TemRO, and CD4+CD45RA+ effector memory T (TemRA) cells.

Serum cytokines

A set of inflammatory (TNF- α , MCP-1, soluble CD40L, and IL-6) and one anti-inflammatory (IL1 receptor antagonist (IL-1Ra)) serum cytokines were measured in serum samples that were kept cool right after harvest and stored at -80 within 4 hours and prevented from freeze-thaw cycles. Multiplex immunoassays (MIA) were used to measure the serum cytokine levels as described previously [44, 45]. Since serum levels of IL-6 were below detection limit, IL-6 was left out of the analysis.

Serum biochemical parameters

Serum levels of C-reactive protein (CRP; mg/L), Rheumatoid factor (RF; IU/mL), Reactive Oxygen Metabolites (ROM; IU/L), and Total Thiol (TTT; $\mu\text{mol/L}$) were measured with a clinical auto-analyzer (Dx5, Beckman-Coulter). Dehydroepiandrosterone Sulphate (DHEAs; $\mu\text{mol/L}$) and 25-Hydroxy-Vitamin-D (VitD; nmol/L) were measured using the immuno-analyser Acces-2 from Beckman Counter.

Statistical analyses

The functional antibody titers were compared between the high and low responders with the Mann Whitney U test. The geometric means with the 95% confidence intervals (CI) are indicated in the graphs. The Chi-square test was used to determine significant differences in patient characteristics between the high and low vaccine responders.

The different immune markers were compared between the high and low responders using the Mann Whitney U test. Furthermore, the group specific geometric mean values of the different immune markers were normalized to z-scores using the geometric means and standard deviation of the total group of 100 participants. The normalized z-scores were displayed on a color scale in the heat maps, ranging from red (below the geometric mean of the total group) to blue (above the geometric mean of the total group). The color darkness is representative of the deviation from the total group geometric mean. For these analyses, SPSS V22.0 and Graphpad Prism V7 were used.

Multivariate redundancy analysis (RDA) was used to assess associations between vaccine responsiveness and the pre-vaccination immune phenotype. The absolute numbers of immune cells, as well as the levels of serum cytokines and biochemical markers were imported in the analysis as biological variables, whereas vaccine responsiveness, age, sex, and CMV were included as explanatory variables. Significance of the explanatory variables was assessed by Monte Carlo permutation testing (MCPT). The p-values as well as the False Discovery Rates (FDR) are given. Biological variables with the highest variation explained by the explanatory variables are depicted in the plots (FitE >15). Canoco5 software for Windows [46] was used to perform this analysis. A p-value of < 0.05 was considered statistically significant.

Results

Participant characteristics

The functional antibody titers of the low and high vaccine responders 28 days post-vaccination are depicted in **Figure 2a**. Although the low responders possess a functional antibody titer below or matching the first quartile of that of the total group, most of these values were above the protection level (functional antibody titer of 8). The fold differences in functional antibody titers between the low and high vaccine responders at 28 days post-vaccination is 141.5, 15.2, and 16.7, for MenC, MenW, and MenY respectively. No difference in pre-vaccination functional antibody titers was found between the low and high responders (**Figure 2b**). The functional antibody titers of part of the low vaccine responders had declined below the protection level at 1 year post-vaccination, whereas all high responders were still highly protected (**Figure 2c**).

Participant characteristics were compared between the low and high vaccine responders (**Table 1**). Only for MenC, the low responders were significantly older as compared to the high responders. Sex distribution, the number of CMV seropositive participants and BMI were comparable between the low and high responders. Moreover, no significant differences in disease incidence (within the last year), medication use (within the last 6 months), the incidence of recent infections (within the last 4 weeks), smoking or physical activity were observed. Not all participants were identified as low or high responder consistently for all meningococcal groups.

Differences in pre-vaccination immune markers between high and low vaccine responders

At first, the absolute numbers of immune cells were compared between the high and low responders for the different meningococcal groups separately (**Figure 3a**). Low responders to MenC possessed significantly higher absolute numbers of naïve Treg ($p=0.033$), CD45RA+CD25^{dim} ($p=0.005$), CD4 naïve ($p=0.021$), CD4 TemRA early ($p=0.024$) and CD8 CM ($p=0.038$) cells as compared to the high responders (**Figure 3a and S. Figure 1a,c,d,f,g**). Moreover, trends towards higher absolute numbers of memory Treg ($p=0.084$) and CD4 TemRA ($p=0.057$) cells were found in the low responders (**Figure 3a and S. Figure 2b and e**). In addition, the low responders to MenC showed lower serum levels of IL-1Ra ($p=0.035$) as compared to the high responders (**Figure 3b and S. Figure 1h and i**), as well as a trend towards lower VitD levels ($p=0.075$) (**Figure 3b and S. Figure 2j**).

Low responders for MenW possessed significantly higher absolute numbers of memory Treg cells ($p=0.039$) (**Figure 3a and S. Figure 2a**) as well as a trend towards lower levels of IL-1Ra ($p=0.057$) (**Figure 3b and S. Figure 2b**) as compared to the high responders.

Table 1. Participant characteristics

	MenC		MenW		MenY	
	Low N=25	High N=27	Low N=46	High N=35	Low N=40	High N=34
Age (95% CI)	59.1* (57.5-60.7)	56.1* (54.5- 57.7)	58.3 (57.1- 59.6)	56.9 (55.5- 58.4)	57.7 (56.4- 59.1)	56.6 (55.0- 58.1)
Males (%)	13 (52%)	16 (59%)	21 (45.6%)	19 (54.3%)	20 (50%)	17 (50%)
CMV seropositive (%)	10 (40%)	17 (63%)	21 (45.6%)	18 (51.4%)	15 (37.5%)	17 (50%)
BMI (95% CI)	24.9 (23.5- 26.3)	24.8 (23.5- 26.2)	25.2 (24.2- 26.4)	25.8 (24.7- 27.0)	24.8 (23.7- 26.0)	26.5 (25.2- 27.8)
Disease in last year (number, %)						
Diabetes type II	0 (0%)	1 (3.7%)	2 (4.3%)	3 (8.6%)	1 (2.5%)	2 (5.9%)
High blood pressure	5 (20.0%)	3 (11.1%)	8 (17.4%)	5 (14.3%)	6 (15%)	8 (23.5%)
Vascular diseases	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2.5%)	0 (0%)
Lung diseases	1 (4.0%)	1 (3.7%)	0 (0%)	4 (11.4%)	2 (5%)	2 (5.9%)
Rheumatic diseases	1 (4.0%)	1 (3.7%)	2 (4.3%)	0 (0%)	2 (5%)	0 (0%)
Gastro-intestinal diseases	0 (0%)	2 (7.4%)	2 (4.3%)	1 (2.9%)	2 (5%)	2 (5.9%)
Other diseases	1 (4.0%)	1 (3.7%)	1 (2.2%)	3 (8.6%)	1 (2.5%)	3 (8.8%)
No serious disease	18 (72%)	19 (70.4%)	35 (76.1%)	26 (74.3%)	31 (77.5%)	24 (70.6%)
Medication last 6 months (number, %)						
Medication for infection #	0 (0%)	4 (14.8%)	3 (6.5%)	4 (11.4%)	2 (5%)	2 (5.9%)
Cholesterol lowering medication	2 (8.0%)	2 (7.4%)	5 (10.9%)	5 (14.3%)	3 (7.5%)	4 (11.8%)
Diabetic medication	0 (0%)	1 (3.7%)	1 (2.2%)	3 (8.6%)	0 (0%)	2 (5.9%)
Blood pressure lowering medication	7 (28.0%)	3 (11.1%)	9 (19.6%)	6 (17.1%)	7 (17.5%)	8 (23.5%)
Immunosuppressive medication	0 (0%)	1 (3.7%)	1 (2.2%)	2 (5.7%)	1 (2.5%)	1 (2.9%)
No medication	18 (72%)	19 (70.4%)	32 (69.6%)	23 (65.7%)	30 (75%)	23 (67.6%)
Infections (number, %)						
Influenza < 4 weeks	0 (0%)	1 (3.7%)	1 (2.2%)	0 (0%)	1 (2.5%)	0 (0%)
Cold < 4 weeks	6 (24.0%)	3 (11.1%)	7 (15.2%)	10 (28.6%)	7 (17.5%)	6 (17.6%)
No infection <4 weeks	19 (76.0%)	25 (92.6%)	39 (85.8%)	26 (74.3%)	33 (82.5%)	29 (85.3%)
Smoking (number, %)						
Cigarette smoking	6 (24.0%)	3 (11.1%)	6 (13%)	3 (8.6%)	6 (15%)	4 (11.8%)
Cigars, pipe smoking	1 (4.0%)	0 (0%)	3 (6.5%)	1 (2.9%)	3 (7.5%)	1 (2.9%)
No smoking	18 (72%)	25 (92.6%)	38 (82.6%)	32 (91.4%)	32 (80%)	30 (88.2%)
Physical activity (number, %)						
Weekly or more	17 (68.0%)	19 (70.4%)	31 (67.4%)	24 (68.6%)	27 (67.5%)	25 (73.5%)
Less than weekly	4 (16.0%)	2 (7.4%)	8 (17.4%)	4 (11.4%)	6 (15%)	4 (11.8%)
No activity	4 (16.0%)	7 (25.9%)	7 (15.2%)	8 (22.9%)	7 (17.5%)	6 (17.6%)

Medication used more than 3 months ago and mainly consisting of corticosteroids and antibiotics.

*p<0.05, significant differences are depicted in bold and underlined. The Chi-square test was used to determine statistical significances.

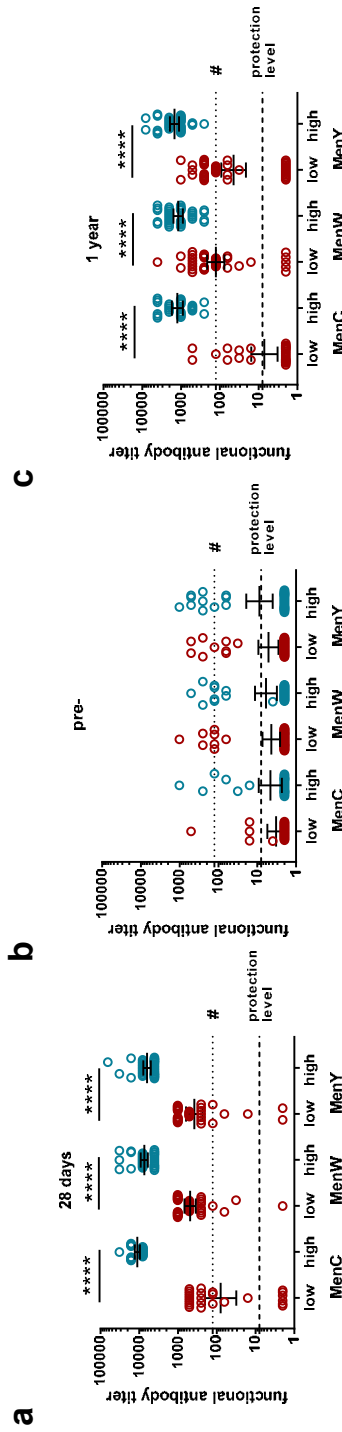


Figure 2. The meningococcal group specific functional antibody titers of the low and high vaccine responders.

The functional antibody titers (a) 28 days post-vaccination, (b) pre-vaccination and (c) 1 year post-vaccination of the low (red) and high (blue) vaccine responders. The protection level is indicated by the lowest dotted line. A more conservative long-term protection level is indicated by the # in the figures. The functional antibody titers were compared between the low and high responders using the Mann Whitney U test. MenC: low N=25, high N=27; MenW: low N=46, high N=35; and MenY: low N=40, high N=34. **** p<0.0001.

Finally, the low responders for MenY had significantly higher absolute numbers of CD45RA+CD25^{dim} cells (p=0.022) as well as lower absolute numbers of natural effector (CD27+IgD+) B-cells (p=0.008), T-cells (p=0.043), CD4 TemRO intermediate cells (p=0.011), CD8 TemRO cells (p=0.027), and CD8 TemRO late cells (p= 0.036) than the high responders (**Figure 3a and S. Figure 3e,b,d,h,i, and k**). Moreover, trends towards lower absolute numbers of total B-cells (p=0.060), CD27+ memory B-cells (Bmem)(p=0.062), CD4RO T-cells (p=0.070), CD4RO early T-cells (p=0.081), and CD8 TemRO intermediate T-cells (p=0.090) (**Figure 3a and S. Figure 3a, c, f, g, and j**) as well as a trend to a lower BMI (p=0.053) (**Figure 3b and S. Figure 3i**) were observed in the low responders.

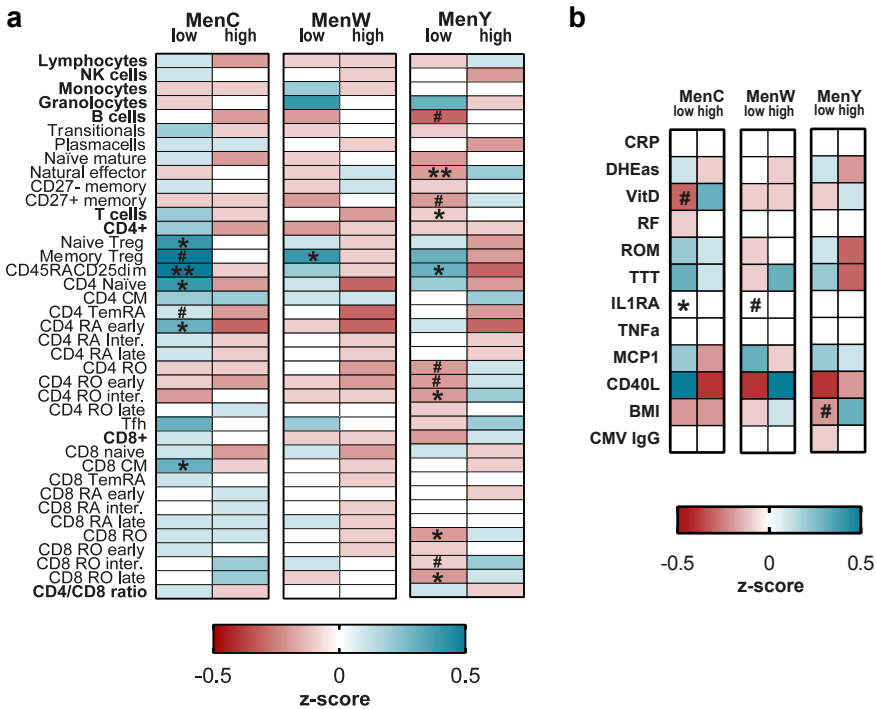


Figure 3. Comparison of pre-vaccination immune markers between the high and low vaccine responders.

Heat maps comparing the absolute immune cells numbers (**a**), serum cytokines and biochemical markers (**b**) between the low and high vaccine responders for the different meningococcal serotypes separately. The absolute immune cell numbers as well as the concentrations of the serum markers were normalized to z-scores using the geometric means. The geometric means of the two groups were compared with the overall group geometric mean and standard deviation. The normalized z-scores are displayed on a colour scale, ranging from red (below the geometric mean of the total group) to blue (above the geometric mean of the total group). The white colour indicates values that are equal to the group geometric mean. The stronger the deviation from the group geometric mean, the darker the colour. The different immune markers were compared between the low and high responders using the Mann Whitney U test. # p<0.1, * p<0.05, ** p<0.01. MenC: low N=25, high N=27; MenW: low N=46, high N=35; and MenY: low N= 40, high N= 34.

Multivariate redundancy analysis (RDA) revealed significant associations between the pre-vaccination immune phenotype and vaccine responsiveness

In order to determine whether high or low vaccine responsiveness at day 28 post-vaccination was significantly associated with all measured pre-vaccination immune markers combined, hereafter called the immune phenotype; a multivariate RDA was performed for the three meningococcal groups separately (**Figure 4a-c**). Overall the included variables (age, sex, CMV, BMI, and vaccine response) explained 14.5%, 10.4%, and 12.0% of the total variation in immune phenotype for MenC, MenW, and MenY respectively.

For MenC and MenY, the variable 'vaccine response' was significantly associated with the immune phenotype (MenC: $p=0.012$, FDR= 0.07 and MenY: $p= 0.028$, FDR= 0.098) (**Figure 4a and c**). As expected, based on the heatmap depicted in **Figure 3a**, for MenW no significant association between vaccine response and immune phenotype was observed ($p=0.068$, FDR= 0.16) (**Figure 4b**).

For the MenC group higher levels of DHEA, TTT, and ROM as well as higher absolute numbers of memory Treg cells, naïve Treg cells, CD45RA+CD25^{dim} cells, CD4 TemRA early cells, CD4 naïve cells, lymphocytes, and CD4 T-cells were strongly associated with low responsiveness, whereas higher levels of IL-1Ra were related with high responsiveness (**Figure 4a**). For MenY, higher levels of DHEA and higher absolute numbers of CD4 naïve, CD45RA+CD25^{dim}, CD8 naïve and naïve Treg cells were strongly associated with low vaccine responsiveness, while high absolute numbers of Bmem cells were linked to high vaccine responsiveness (**Figure 4c**). In addition, CMV seropositivity was significantly associated with the immune phenotype (**Figure 4a**: $p=0.098$, FDR= 0.23, **Figure 4b**, $p=0.002$, FDR= 0.014, and **Figure 4c**, $p=0.002$, FDR= 0.014). In these analyses, CMV seropositivity was associated with higher absolute numbers of CD4RO late, CD4RA late, and CD8RA late T-cells, and not related to either low or high vaccine responsiveness. Of note, the explanatory variables BMI, age, and sex were not significantly associated with the immune phenotype.

Differences in immune cell ratios in the CD4 T-cell compartment between the high and low vaccine responders

In relation to the mentioned findings of higher naïve CD4 T-cells and memory Treg cells in the low vaccine responders, we determined whether the ratio of naïve to memory cells, as well as the ratio of Treg to effector cells in the CD4 T-cell compartment was different between the low and high vaccine responders (**Figure 5**). No difference in the ratio between the total Treg cells and the number of CD4 TemRO T-cells was observed (**Figure 5a**), whereas the ratio of memory Treg cells to CD4 TemRO T-cells was significantly higher in the low responders for MenC and MenY as compared to the high responders (**Figure 5b**).

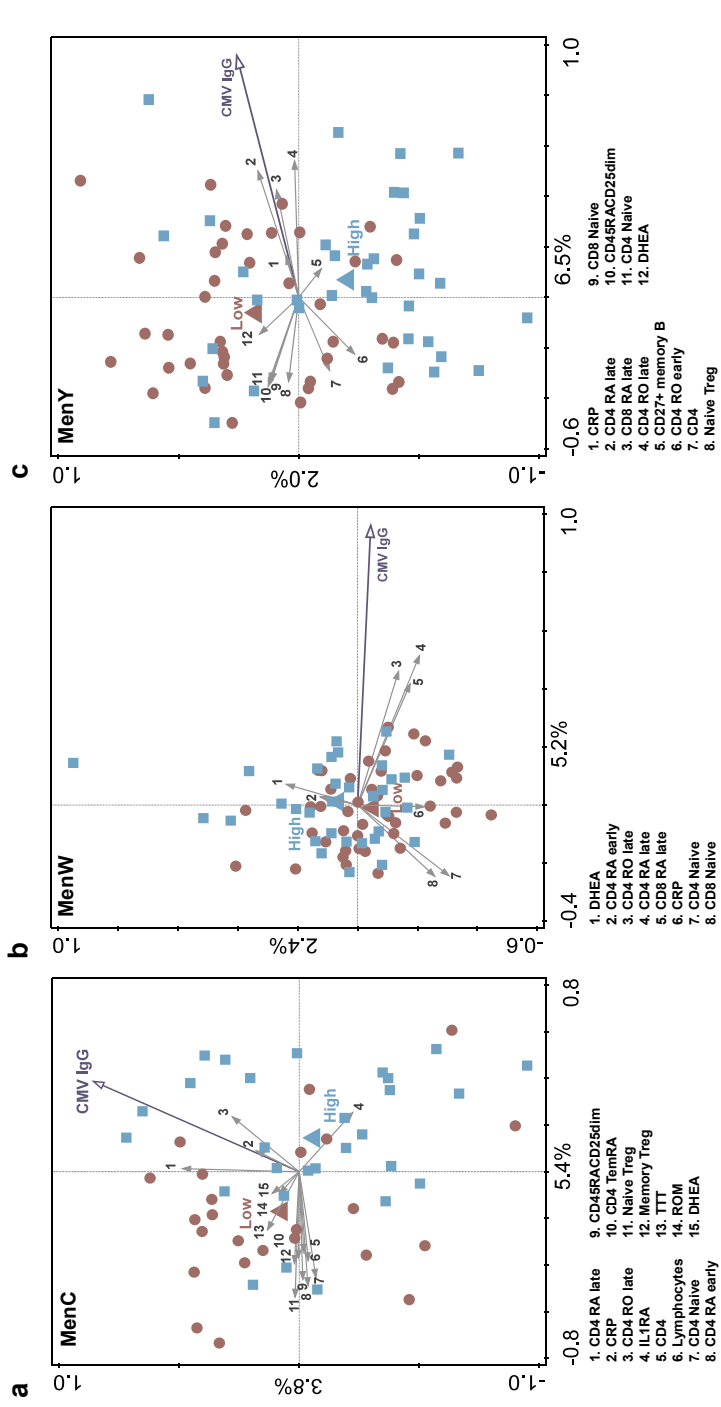


Figure 4. Redundancy analysis (RDA) assessing the association between the pre-vaccination immune phenotype and the meningococcal vaccine response.

RDA of samples collected 28 days post-vaccination for MenC (a), MenW (b), and MenY (c). Low vaccine responders are shown in red circles; High responders are shown in light blue squares. The first and second ordination axes are plotted, including the percentages of explained variation. Overall, 14.5%, 10.4%, and 12.0% of the variation in the datasets was explained for MenC, MenW, and MenY respectively. The vaccine response variable (i.e. low or high responder) was significantly associated with the immune phenotype for MenC ($p=0.012$, $FDR=0.07$) and MenY ($p=0.028$, $FDR=0.098$), but not for MenW ($p=0.068$, $FDR=0.16$). CMV was significantly associated with the immune composition for MenW ($p=0.002$, $FDR=0.014$) and MenY ($p=0.002$, $FDR=0.014$) but not for MenC ($p=0.098$, $FDR=0.23$). Other environmental variables tested (i.e. BMI, age, and sex) did not significantly influence the variation in the dataset. The biological variables with the highest variation explained by the explanatory variables are depicted in the plots (Fit >15). The length of the arrows relates to the strength of the association.

In addition, the low responders for MenC and MenY possessed a significantly higher ratio of the naïve CD4 T-cells and the total CD4 memory cells (**Figure 5c**), as well as a higher ratio of the post thymically expanded CD45RA+CD25^{dim} cells and the total CD4 memory cells (**Figure 5d**). Thus, the higher numbers of both naïve CD4 T-cell subsets and Tregs as seen in low responders at baseline (pre-vaccination) gives rise to clear compositional changes in the peripheral CD4 T-cell compartment.

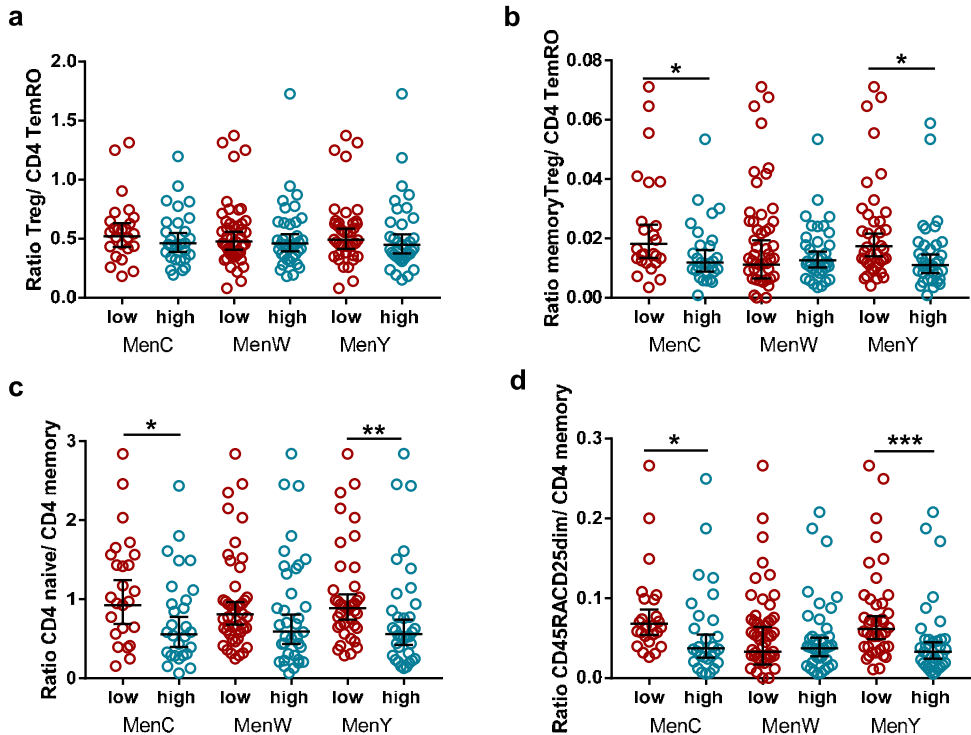


Figure 5. Comparison of immune cell ratios in the CD4 T-cell compartment between the high and low vaccine responders.

Comparison of the Treg/TemRO ratio (**a**), memory Treg/TemRO ratio (**b**), CD4 naïve/ CD4 memory ratio (**c**), and CD45RA+CD25^{dim}/ CD4 memory ratio (**d**) between the low (red) and high (blue) vaccine responders for the different meningococcal groups separately. The total CD4 memory cells were determined as the sum of the CD4 CM, CD4 TemRO, and CD4 TemRA cells. The low and high responders were compared with the Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Discussion

In this explorative study, we investigated whether the pre-vaccination immune phenotype was significantly different between the middle-aged adults being either low or high responder after a primary meningococcal vaccination. Interestingly, the numbers of several CD4 T-cell subsets differed between the low and high vaccine responders. More specifically, low vaccine responders possessed higher numbers of naïve Treg, memory Treg, naïve CD4 cells, and the subset of post thymically expanded CD4+CD45RA+CD25^{dim} T-cells, whereas high responders showed high levels of serum IL-1Ra. These results suggest that the pre-vaccination CD4 signature may be used to identify middle-aged adults who are potential non/low responders to a primary meningococcal vaccine. Identification of these middle-aged adults may help improve timely vaccination strategies, since vaccination schemes, doses, and adjuvant use might be adapted to improve the vaccine responsiveness in these adults.

Numbers of Treg cells are known to increase with advancing age and suggest elevated immune suppression in older adults, although the exact functionality of these Treg cells in ageing individuals is still under investigation [18, 47]. Accordingly, high numbers of Tregs were previously associated with low VZV vaccine responses in nursing home elderly [17]. Within our study, high absolute numbers of both naïve and memory Tregs were associated with low vaccine responsiveness. Naïve Treg cells express CCR7 enabling these cells to migrate to lymphoid organs, whereas memory Tregs home to the sites of inflammation along with effector T-cells [48, 49]. Accordingly, our results suggest enhanced suppression of the vaccine response both in the lymphoid organs as well as the site of vaccination in low responders. Elevated numbers of Treg cells might suppress T-cell responses towards the tetanus carrier in this conjugated meningococcal vaccine and/or inhibit B-cell responses directly [50]. Our results confirm previous findings of high numbers of memory Treg cells in low vaccine responders to influenza [20], whereas we are the first showing an association between low vaccine responsiveness and high numbers of naïve Tregs. Of importance, we observed a higher ratio of memory Treg to effector CD4 T-cells in the low responders, suggesting a shift in the Treg/Teffector balance, as previously observed with advancing age [20].

The consistent association found between high numbers of CD4 naïve and the post thymically expanded CD4+CD45RA+CD25^{dim} cells and a higher ratio of these naïve cells to the memory CD4 T-cell compartment with low vaccine responsiveness was unexpected, since a naïve T-cell repertoire is generally accepted to be beneficial in older adults [8, 10]. Previously, the CD4+CD45RA+CD25^{dim} subset was found to represent a broad and functional reservoir of naïve CD4 T-cells, although some contraction in certain TCR V β families was observed in comparison to naïve CD4 T-cells that recently left the thymus [51]. Since no prior studies were available that linked the numbers of CD4+CD45RA+CD25^{dim} cells to

vaccine responses, our findings indicate the necessity for further research into repertoire size and functionality of these cells. However, the increase in memory Treg cells might be the dominant factor in predicting vaccine response, overruling the presence of a capable naïve CD4 T-cell repertoire.

In line with the different studies investigating biomarkers for influenza and hepatitis B vaccine responses in the elderly [16, 22, 24], we observed trends towards lower numbers of switched memory CD27+ B-cells in the low responders. In contrast, we did not find increased numbers of CD27- memory B-cells in the low responders, as reported by others in vaccine recipients above 65 years of age [22]. In addition, low numbers of natural effector CD27+IgD+ B-cells, cells that were previously found to decrease with age [23], were observed in the low vaccine responders. Since we previously described that IgM is essential in the antibody functionality against the meningococcal groups [4], lower numbers of these natural effector cells, mainly producing IgM, likely explain the lower functional antibody titers.

Currently, effects of latent CMV infection on vaccine responses is controversial [52]. Despite the clear associations between CMV seropositivity and higher numbers of late differentiated T-cells, we did not find an association between CMV seropositivity and meningococcal vaccine responsiveness. As this meningococcal vaccine response is primarily B-cell mediated, the effect of CMV might be limited. Hence, the effects of CMV on T-cell mediated vaccine responses, i.e. to influenza and VZV vaccination should be further elucidated. Although frequently suggested by others [53-56], we did not observe any effects of sex and BMI on the vaccine responses. Moreover, the effect of chronological age was inconsistent, although low responders to MenC were significantly older than high responders.

Of note, high levels of IL-1Ra were found in the high responders. IL-1Ra is known as the receptor antagonist of the IL-1 family, executing anti-inflammatory functions [57, 58]. These results possibly suggest that IL-1Ra acts as an anti-inflammatory counterpart of the 'inflammageing' process. Of note, a trend towards high levels of MCP-1 was found in the low responders. MCP-1 is classified as a pro-inflammatory cytokine, attracting monocytes to the site of inflammation, and serum levels were shown to increase with age [59]. Consequently, our findings may suggest a higher pro-inflammatory state in the low responders [32, 33]. Nevertheless, serum levels of other inflammatory cytokines, such as IL-6 and TFN- α were still low in all participants.

Of importance, associations with the immune phenotype were primarily found between the extremes in the vaccine response, being either low or high responders. The intermediate group showed high variability of immune markers. In addition, our results may imply meningococcal group specific associations between the pre-vaccination immune phenotype and vaccine responsiveness. Noteworthy, the difference in functional antibody titers between the low and high vaccine responders was largest for MenC, possibly explaining

the higher numbers of immune parameters found associated with the vaccine response for this meningococcal group. Moreover, the participants that were classified as low or high responder did not completely overlap between the different meningococcal groups. This might be explained by the structural differences in the meningococcal polysaccharides by which different epitopes will induce distinct immune responses [60], also shown previously for several pneumococcal conjugated polysaccharides [61]. Furthermore despite similar pre-vaccination functional antibody titers in the low and high vaccine responders, differences in numbers of meningococcal group-specific memory B-cells in the bone marrow could have been present due to historical contacts [62] and affect the vaccine response. Nevertheless, the finding of meningococcal group specific associations between vaccine responsiveness and immune phenotype is remarkable and requires further research.

An important strength of this study is the ability to compare multiple antigens and multiple immune parameters within the same group of participants. Also, the primary nature of the vaccination allowed us to explore the use of biomarkers, without the strong interference of pre-vaccination meningococcal immunity, as often seen in other studies. Unfortunately, the presence of pre-vaccination immunity in some participants did interfere with the long-term functional antibody titers (after 1 year). Consequently, we were not able to investigate the associations between pre-vaccination immune phenotype and the long-term vaccine responsiveness, since exclusion of participants with detectable pre-vaccination functional antibody titers dramatically reduced the power of the statistical analysis. In addition, information on the genetic background of the participants could have added to the predictive factors in our analysis, since several studies found associations between genetic signatures and vaccine responsiveness [24, 30, 31].

Future studies, analyzing large cohorts, using different vaccines, and using similar biomarker analyzing techniques are warranted to validate the use of the suggested biomarkers. Hence, systems vaccinology, combining data on genetic background and environmental factors such as diet, stress and infections and even microbiome composition is a promising tool to discover these predictive biomarkers [63]. Moreover, future research should compare the suitability of biomarkers in cohorts of different ages, in order to determine the predictive values of these markers over the entire lifespan.

In conclusion, our explorative biomarker analysis suggests several associations between the pre-vaccination immune phenotype and vaccine responsiveness after primary meningococcal vaccination in middle-aged adults. In general, an altered CD4 T-cell signature, involving high absolute numbers of naïve Treg, memory Treg, naïve CD4 T-cells and CD45RA+CD25^{dim} T-cells might be used as a predictive immune phenotype for low vaccine responsiveness in middle-aged adults. Accordingly, these findings support the development of vaccination strategies to enhance the memory immunity before reaching old age, in the rapidly ageing population.

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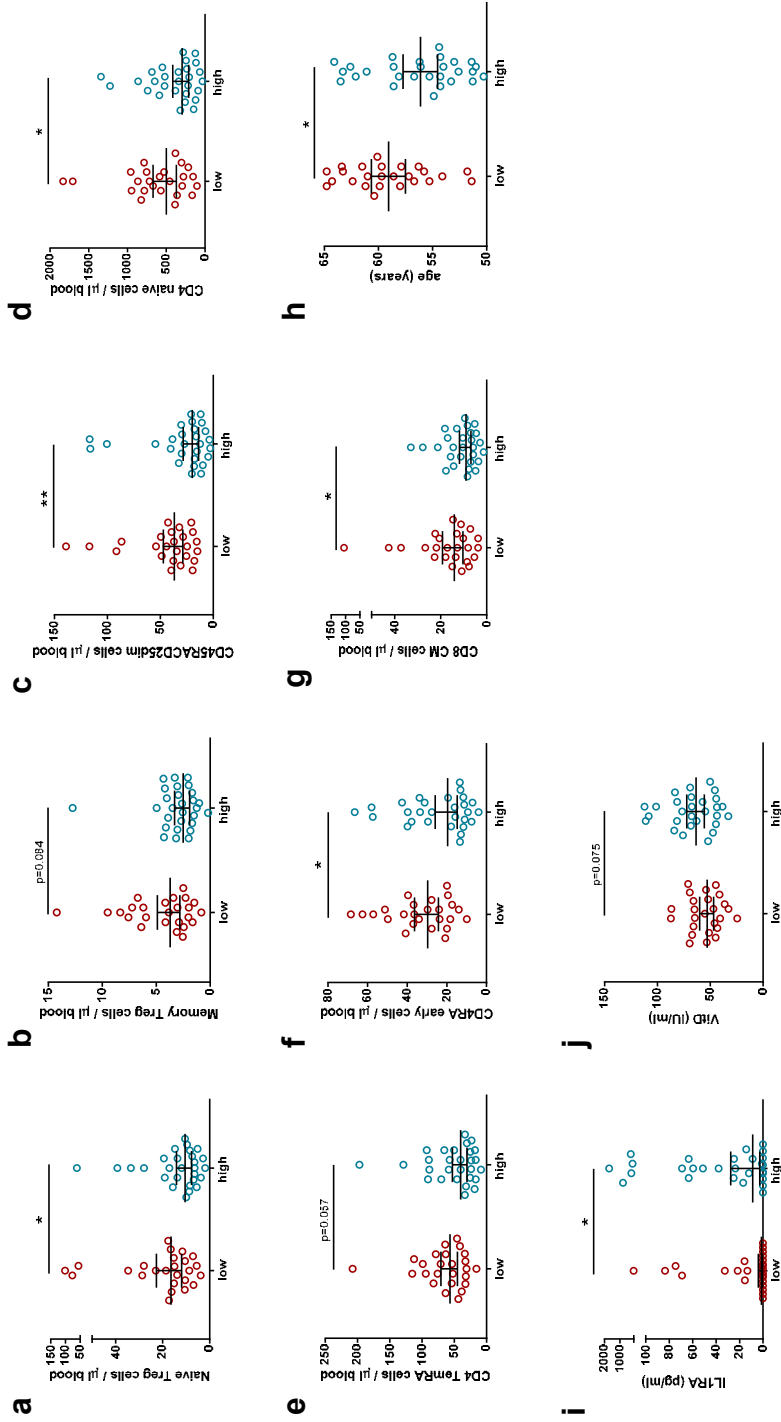
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Supplementary information

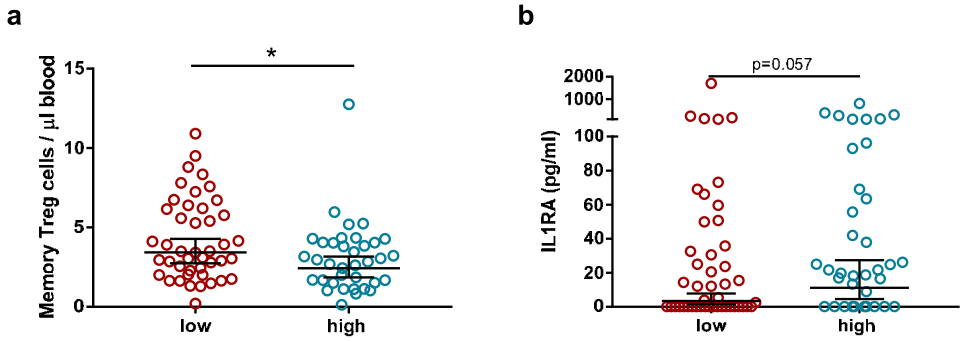
Supplementary Table 1. Phenotypical definitions of the immune cells subsets

Subset	Phenotype definition
Monocytes	SSC ^{inter} CD45+
Granulocytes	SSC ^{high} CD45+
Lymphocytes	SSC ^{low} CD45+
NK cells	SSC ^{low} CD45+CD3-CD16+ or CD56+
B cells	SSC ^{low} CD45+CD19+
Transitional	SSC ^{low} CD45+CD27-CD38+
Plasma cells	SSC ^{low} CD45+CD27+CD38+
Naïve mature	SSC ^{low} CD45+CD27-IgD+CD38 ^{dim}
Natural effector	SSC ^{low} CD45+CD27+IgD+CD38 ^{dim}
CD27- memory	SSC ^{low} CD45+CD27-IgD-CD38 ^{dim}
CD27+ memory	SSC ^{low} CD45+CD27+IgD-CD38 ^{dim}
T cells	SSC ^{low} CD45+CD3+
CD4 T-cells	SSC ^{low} CD45+CD3+CD4+
Naïve Tregs	SSC ^{low} CD45+CD3+CD4+CD45RO-CD25 ^{int}
Memory Tregs	SSC ^{low} CD45+CD3+CD4+CD45RO+CD25 ^{high}
CD45RA+CD25 ^{dim}	SSC ^{low} CD45+CD3+CD4+CD45RO-CD25 ^{dim}
CD4 naïve	SSC ^{low} CD45+CD3+CD4+CD45RO-CCR7+
CD4 CM	SSC ^{low} CD45+CD3+CD4+CD45RO+CCR7+
CD4 TemRA	SSC ^{low} CD45+CD3+CD4+CD45RO-CCR7-
CD4 TemRA early	SSC ^{low} CD45+CD3+CD4+CD45RO-CCR7-CD27+CD28+
CD4 TemRA intermediate	SSC ^{low} CD45+CD3+CD4+CD45RO-CCR7-CD27-CD28+
CD4 TemRA late	SSC ^{low} CD45+CD3+CD4+CD45RO-CCR7-CD27-CD28-
CD4 TemRO	SSC ^{low} CD45+CD3+CD4+CD45RO+CCR7-
CD4 TemRO early	SSC ^{low} CD45+CD3+CD4+CD45RO+CCR7-CD27+CD28+
CD4 TemRO intermediate	SSC ^{low} CD45+CD3+CD4+CD45RO+CCR7-CD27-CD28+
CD4 TemRO late	SSC ^{low} CD45+CD3+CD4+CD45RO+CCR7-CD27-CD28-
Tfh	SSC ^{low} CD45+CD3+CD4+CXCR5+
CD8 T-cells	SSC ^{low} CD45+CD3+CD8+
CD8 naïve	SSC ^{low} CD45+CD3+CD8+CD45RO-CCR7+
CD8 CM	SSC ^{low} CD45+CD3+CD8+CD45RO+CCR7+
CD8 TemRA	SSC ^{low} CD45+CD3+CD8+CD45RO-CCR7-
CD8 TemRA early	SSC ^{low} CD45+CD3+CD8+CD45RO-CCR7-CD27+CD28+
CD8 TemRA intermediate	SSC ^{low} CD45+CD3+CD8+CD45RO-CCR7-CD27+CD28-
CD8 TemRA late	SSC ^{low} CD45+CD3+CD8+CD45RO-CCR7-CD27-CD28-
CD8 TemRO	SSC ^{low} CD45+CD3+CD8+CD45RO+CCR7-
CD8 TemRO early	SSC ^{low} CD45+CD3+CD8+CD45RO+CCR7-CD27+CD28+
CD8 TemRO intermediate	SSC ^{low} CD45+CD3+CD8+CD45RO+CCR7-CD27+CD28-
CD8 TemRO late	SSC ^{low} CD45+CD3+CD8+CD45RO+CCR7-CD27-CD28-



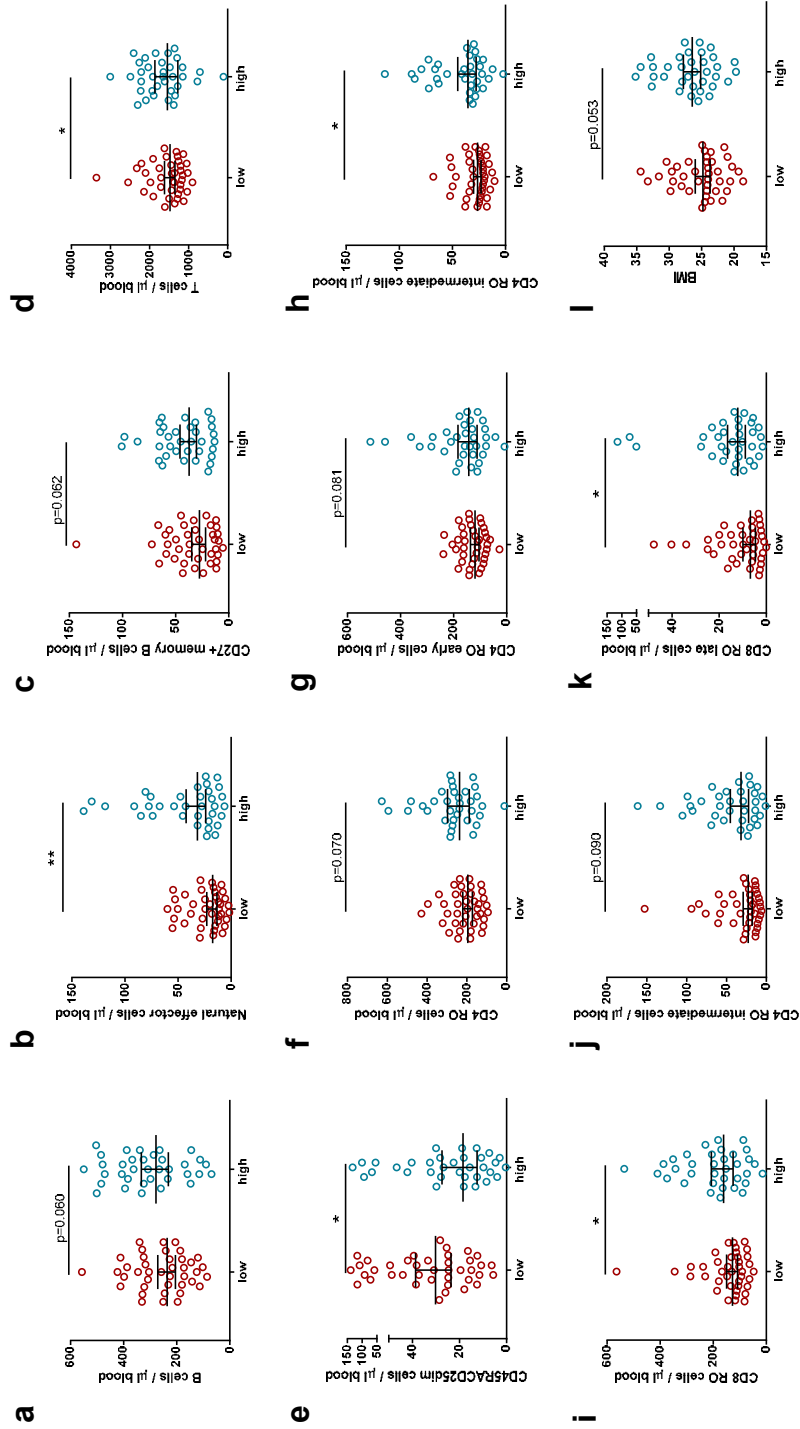
Supplementary Figure 1. Analysis of differences in immune markers between high and low responders for MenC.

The differences in absolute numbers of naive Treg (a), Memory Treg (b), CD45RA+CD25dim (c), CD4 naïve (d), CD4 TemRA (e), CD4 TemRA early (f), CD8 CM (g), age (h), and the levels of IL-1RA (i) and VtID (j) between the low (red, N=25) and high (blue, N=27) responders for MenC. The Geometric means with 95% CI interval are indicated in the graphs. The low and high responders were compared for the different immune markers using the Mann Whitney U test. Trends are given as p-values. * p<0.05, ** p<0.01.



Supplementary Figure 2. Analysis of differences in immune markers between the high and low responders for MenW.

The difference in absolute numbers of memory Treg (a) and levels of IL-1Ra (b) between the low (red, N= 46) and high (blue, N=35) responders for MenW. The geometric mean with 95% CI interval is indicated in the graphs. The early low and high responders were compared for the different immune markers using the Mann Whitney U test. Trends are given as p-values. * p<0.05.



Supplementary Figure 3. Analysis of differences in immune markers between the high and low responders for MenY.

The difference in absolute numbers of B-cells (**a**), Natural effector B-cells (**b**), CD27+ memory B-cells (**c**), T-cells (**d**), CD45RA⁺CD25^{dim} cells (**e**), CD4 TemRO cells (**f**), CD4 TemRO early cells (**g**), CD4 TemRO intermediate cells (**h**), CD8 TemRO cells (**i**), CD4 TemRO intermediate cells (**j**), CD8 TemRO late cells (**k**) and BMI (**l**) between the low (red, N= 40) and high (blue, N= 34) responders for MenY. The geometric mean with 95% CI interval is indicated in the graphs. The low and high responders were compared for the different immune markers using the Mann Whitney U test. Trends are given as p-values. $*$ $p<0.05$. $**$ $p<0.01$.