Natural and vaccine derived immunity against the human papillomavirus

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CHAPTER 6

CHARACTERIZATION OF THE EARLY CELLULAR IMMUNE RESPONSE INDUCED BY HPV VACCINES AND ITS RELATION TO LONG-TERM HPV-SPECIFIC IMMUNITY

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ABSTRACT

Introduction Current human papillomavirus (HPV) vaccines consist of virus-like particles (VLPs) which are based on the L1 protein, but they are produced by different expression systems and use different adjuvants. We performed in-depth immunophenotyping of multiple innate and adaptive immune cells after bivalent versus nonavalent HPV vaccines.

Method Twenty pre-menopausal HPV-seronegative women were enrolled and randomized to receive three-doses of either the bivalent or the nonavalent HPV vaccine. Blood samples were collected at multiple time points from baseline up to 7 months after first vaccination. Four different Euroflow flow cytometry antibody panels were used to monitor >250 immune cell subsets. HPV-specific memory B- and T cells were determined by ELISPOT and HPV-specific antibody levels were measured by a VLP-based multiplex immunoassay.

Results The numbers of plasma cells expanded in the first week after both primary and tertiary vaccination in both cohorts. HPV16 and HPV18-specific antibody levels and memory B and T cell responses were higher in the bivalent cohort than in the nonavalent vaccines one month post third vaccination. For HPV31 and HPV45-specific antibody levels this pattern was reversed. Monocytes showed a clear expansion one day after vaccination in both cohorts but were significantly higher in the bivalent vaccine cohort. Large heterogeneity in responses of the other cell subsets was observed between donors.

Conclusion This pilot study showed a consistent response of monocytes and plasma cells after vaccination and a considerable variation in other circulating immune cells in both types of HPV vaccines between donors.

INTRODUCTION

A HPV infection is one of the most common sexually transmitted infections worldwide. Already over 200 different subtypes have been identified of which 15 are classified as oncogenic and are an important cause of anogenital and oropharyngeal cancers, but most importantly of cervical cancer. HPV16 and 18 are associated with about 70% of all cervical cancer cases, and 25% are associated with closely related HPV types within the groups A9 (HPV16-like: 31,33,35,52,58) and A7 (HPV18-like; 39,45,59,68) [1, 2]. Globally, around 800 women die of cervical cancer every day [3].

Currently, there are three licensed, highly efficacious prophylactic vaccines on the market providing protection against two (bivalent), four (quadrivalent), and nine (nonavalent) persistent HPV infections, and to some degree there is cross-protection against phylogenetically related non-vaccine types. All vaccines comprise virus-like particles (VLPs) which are based on the major HPV capsid protein L1. The antigens of the vaccines are produced in different expression systems; a baculovirus expression system is used for the bivalent vaccine and a yeast expression system for the quadrivalent and nonavalent vaccines. Moreover, the vaccines use different types of adjuvants. The bivalent vaccine is formulated with AS04, containing aluminum hydroxide salts and the TLR4 agonist MPL (3-O-deascyl-4'-monophosphoryl lipid A). The quadrivalent and nonavalent vaccines consist of aluminum salts as an adjuvant [4].

Several studies have investigated the differences in immunological responses to these different vaccines, and especially comparing the bivalent with the quadrivalent vaccine [5-9]. The majority of this work has focused on antibody responses, as they are thought to be the key mediators of protection against infection [10]. All three vaccine have been shown to induce robust antibody responses against the various vaccine types [9, 11, 12], but the bivalent vaccine has been shown to induce higher levels of HPV16/18-specific serum antibodies [6, 13] and more robust B cell responses [7]. The AS04 adjuvant is suggested to be of importance for this higher immunogenicity observed in bivalent vaccine recipients, as it is thought to induce a better Th1 response [14, 15].

To the best of our knowledge, innate cellular responses after HPV vaccination have not been studied so far. Insight in these responses would aid the interpretation of the different working mechanisms of the vaccines, and the induced adaptive responses observed.

The aim of this study was to increase our knowledge of early cellular immune reactions after vaccination with either the bivalent or the nonavalent HPV vaccine, and their possible involvement in/possible relation to HPV-specific long-term memory formation. Therefore, we investigated the kinetics of circulating innate and adaptive immune cell subsets by in-depth phenotyping after the first and last vaccination. Kinetics of circulating cells were related to the induction of long-term antibody and cellular memory responses upon vaccination.

METHODS

Study population and procedures

Participants were recruited among pre-menopausal female personnel of the Dutch National Institute for Public Health and the Environment (RIVM). Potential participants were invited to a

pre-study visit and asked to donate a finger prick blood sample to measure the presence of anti-HPV antibodies. During that visit they had the opportunity to ask questions about the study. HPV-seronegative (HPV16/18/31/45) women who were willing to participate were invited for further study consultation and signing the informed consent form according to the Declaration of Helsinki. The study was approved by the Medical Ethical Committees United, Nieuwegein, the Netherlands (study number: NL69015.100.19). Inclusion and exclusion criteria are listed in Supplementary Table 1.

Twenty women were enrolled and randomized in a 1:1 ratio to receive either the bivalent (GSK, Rixensart, Belgium) or the nonavalent (Merck, Sharp &Dohme, Kenilworth, NJ) HPV vaccine according to a three-dose schedule (0, 2 and 6 months). Whole blood and serum samples were collected at baseline (day 0) and at pre-defined time points following vaccination. Following primary vaccination, an initial cohort of five volunteers donated blood at eight visits (days 0 (baseline), 1, 2, 3, 6, 7, 10, and 14) to determine the most optimal sampling scheme (Figure 1A) for the remaining 15 donors (days 0, 1, 3, and 7). Then, all volunteers received the first and second booster vaccination and donated samples at day 80, 180 (booster baseline), 181, 183, 187 and 208 (Figure 1B).

Immunophenotyping by flow cytometry

PB-EDTA samples were processed within 2 hours from blood collection. Four different Euroflow flow cytometry antibody panels were used to monitor kinetics of over 250 immune cell subsets in peripheral blood; a dendritic cell (DC)-monocyte tube (MDC; Van der Pan ***et al.***,

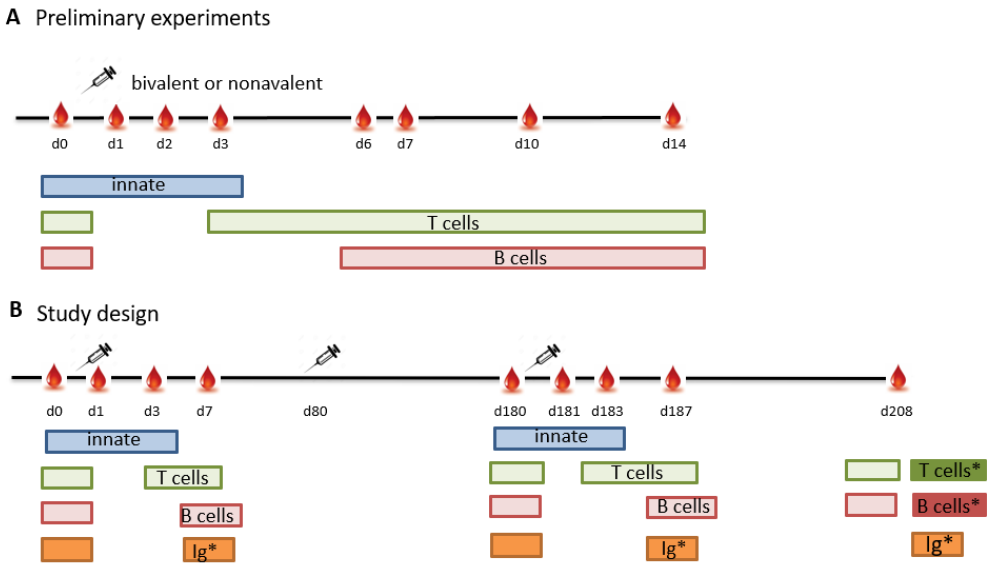


Figure 1 Study design; Twenty healthy HPV16/18/31/33/45/52/58 seronegative adult pre-menopausal women received either the bivalent or nonavalent HPV vaccine according to a three-dose schedule. (A) In the preliminary experiments a blood sample was taken just before vaccination (day 0), and after the first vaccination at days 1, 2, 3, 6, 7, 10 and 14 (B) In the study a blood sample was drawn just before (day 0), and after the first vaccination at days 1, 3, 7. At two months a second vaccination was given. Just before (day 180), an after the 3rd vaccination also blood samples were drawn at days 181, 183, 187 and 208. The colors indicate a which timepoints which immune components were studied, blue: innate cells, green: T cells, red= B cells, orange- IgG antibodies. *= HPV16/18/31/45-specific.

manuscript in preparation), a CD4 T cell tube (CD4T) [16], a CD8 cytotoxic T cell tube (CYTOX tube) and a B cell and plasma cell tube (BIGH)[17, 18]. Additionally, Perfect Count microspheres™ (Cytognos) were used according to the EuroFlow protocol (www.EuroFlow.org) for precise enumeration of cell numbers using the Perfect Count tube (PCT). The CD4 T-cell, CYTOX and the PCT were directly stained on whole blood, using either 100µl (T cells) or 50µl (PCT) of peripheral blood. For the CD4 T cell and CYTOX tube, 100µl PB was stained for 30min at RT in the dark with the corresponding antibody panels. After washing, 100 µl of Reagent A (Fix & Perm, Nordic MUbio, Susteren, the Netherlands) was added and incubated for 15 min at RT in the dark. Cells were washed and 100µl of Reagent B (Fix&Perm™ Nordic MUbio, Susteren, the Netherlands) and intracellular antibodies (Granzyme B, CD154) were added and incubated for 15 min at RT in the dark. After washing, cells were resuspended in 200µl PBS and acquired immediately or were stored at 4°C (max.1 hour) and measured on LSR-Fortessa or Fortessa X20 flow cytometers (BD Biosciences) (stain-lyse-wash protocol, followed by intracellular staining; protocols available at www.EuroFlow.org). The samples for BIGH and MDC tubes were processed according to the bulk lysis protocol (available at www.EuroFlow.org). Briefly, NH_4Cl was added to 1.5ml (WBC > $8x10^6$/ml) or 2.0 ml (WBC <$8x10^6$/ml) of PB to a final volume of 50mL, and was incubated for 15min on the roller bank at RT. Samples were centrifuged for 10 min at 800g, and washed twice in PBS/0.2%BSA/2mMEDTA/0.09%NaAz. Then, $10*10^6$ cells were stained for 30 min in the dark at RT with corresponding antibody panels (NB: the MDC tube was incubated rolling in the dark). The BIGH staining was followed with the Fix & Perm procedure for intracellular staining with Ig subclasses only. For the MDC tube, which did not require intracellular staining, 2mL of BD lyse (BD FACS™ Lysing solution, BD biosciences) was added, incubated for 10min in dark at RT, and washed. Then, cells were resuspended in 500uL of PBS and immediately measured on one of the BD Fortessa flow cytometers or after storage at 4°C for max 1h.

Flow cytometers were calibrated daily according to the EuroFlow guidelines. In short, the photomultiplier tube (PMT) voltages of the flow cytometer were set using BD™ Cytometer Setup and Tracking (CS&T) beads (BD Biosciences) and SPHERO™ Rainbow calibration particles (Cytognos), as previously described [19, 20]. For data analysis, Infinicyt software v 2.0 (Cytognos, Salamanca, Spain) was used.

The Euroflow antibody panels of the different tubes were designed for the PERISCOPE consortium and are elaborately described elsewhere, for the MDC tube (van der Pan ***et al.***, manuscript in preparation), for CD4 [16], the CYTOX and the B cells tube (patent file in preparation: N2023163, filing date 5 Nov 2019) [17]. The antibody panels and corresponding analysis strategies were designed for the PERISCOPE consortium [17]. Both absolute cell counts (cells/ µl) and ratios over baseline were used through the manuscript. Ratios at days 1, 3 and 7 were calculated over the pre-vaccination baseline (day 0) and ratios at days 181, 183, 187 and 208 were calculated over the pre-second booster baseline (day 180).

Detection of memory B cells by ELISPOT

From 18ml of blood collected one month post third vaccination, peripheral blood mononuclear cells (PBMCs) were immediately isolated and stored at -135◦C until analysis. After thawing, B cells were purified from PBMCs by a CD19+ selection kit (StemCell Technologies, Vancouver,

Canada) and stimulated polyclonally with CPG and cytokines for five days as described previously [21]. HPV16, HPV18, HPV31 and HPV45-specific ELISPOT-assays were performed by coating multiscreen-IP plates (Millipore, Burlington, MA) with PBS containing 20 ug/ml HPV16, 18, 31 or 45 VLPs. A suspension of $1x10^5$ B cells was added per antigen in triplicate per participant. Tetanus toxoid (7 flocculation units/ml in PBS) and PBS-coated wells were included as positive- and negative controls, respectively.

For detection of antibody-producing cells as spots, alkaline-phosphatase conjugated goat anti-human IgG was added in combination with BCIP/NBT substrate (Sigma Aldrich, Saint Louis, MI). Spots were analyzed using an Immunospot reader and software (CTL Immunospot S6 Ultra-V Analyzer, Bonn, Germany). Geometric mean (GM) of spot numbers in the PBS-coated wells per participant were subtracted from all antigen-specific spot numbers per participant. GM numbers of HPV-type-specific memory B cells were expressed per 10^5 B cells. When no HPV-specific spots were detected in any of the wells, values were $<0.2/10^5$ B cells and set to value of 0.1.

Detection of IFN-Y producing cells by ELISPOT

Numbers of HPV-specific IFN-γ-producing cells were measured by ELISPOT. PBMCs were stimulated with VLPs; 4 μg/mL(HPV16, HPV31 and HPV45) and 2 μg/mL(HPV18), in triplicate, in $3x*10^5$ cells/well in AIMV medium (Gibco, Waltham, MA) containing 2% human AB-serum (Harlan, Indianapolis, IN), for 4 days at 37°C and 5% CO_2. Unstimulated and lectin-stimulated cells served as negative and positive controls, respectively. Subsequently, the number of IFN-γ-producing cells specific for HPV16, HPV18, HPV31 and HPV45 was measured using ELISPOT-assays as described previously [22, 23]. Spot numbers were counted using an Immunospot reader (version V3.0) and software (version V6.1) (CTL Immunospot S6 Ultra-V Analyzer, Bonn, Germany). Geometric mean (GM) spot numbers of unstimulated cells per participant were subtracted from the HPV-type-specific spot numbers per participant. GM numbers of HPV-type-specific IFN-γ producing cells were expressed per $1*x10^5$ PBMCs.

HPV-specific antibody levels

HPV16/18/31 and 45 specific IgG and IgA antibody levels in serum at day 7, 14, 180, 187 and in plasma at day 208 after the first vaccination were determined by using a VLP-based multiplex-immunoassay. All VLPs used in this study were provided at cost part by GSK. The VLP-based multiplex has been described elsewhere in detail [24]. In short, sera were incubated with HPV-specific VLP-conjugated beads (Bio-Rad Laboratories, Hercules, CA). HPV-specific IgG antibodies were detected using R-phycoerythrin (PE) conjugated goat anti-human IgG (Jackson Immunoresearch, West Grove, PA). The 'in-house' control sera and a standard (IVIG Baxter, Utrecht, the Netherlands) were used on each Multiscreen HTS filter plate (Millipore, Burlington, MA). For IgA, a 1/200 dilution of R-PE conjugated goat anti-human IgA (Jackson Immunoresearch, West Grove, PA) was used. HPV-specific antibodies were analyzed using the Bioplex-system 200 with Bioplex-software (Bio-Rad Laboratories, Hercules, CA). IgG antibody levels were expressed in Luminex units (LU) per mL. Semi-quantitative IgA antibody concentrations were expressed in mean fluorescence intensity (MFI).

The presence HPV16, 18, 31 and 45 specific IgG subclasses (IgG1, IgG2, IgG3, IgG4), was determined at day 187. Analysis was performed as described above, by using IgG-isotype-specific mouse anti-human R-PE conjugated secondary antibodies used in 1/500 dilution (IgG1), 1/100

(IgG2-4) (SouthernBiotech, Birmingham, AL). Distributions of IgG-subclasses in percentages were calculated using median fluorescent intensity (MFI) of the IgG subclasses separately in relation to the MFI of the sum of all subclasses, which was set at 100%.

Statistical analysis

Univariate summaries comprise geometric mean concentrations (GMC) with their 95% confidence intervals (CI). Bivariate summaries comprise Spearman correlations and the permuted version of the Spearman test for pairs of immune cells at each time point. Multivariate exploratory methods comprise principal component analyses (PCA) performed either per time point or longitudinally.

The permuted version of the Wilcoxon-Mann-Whitney non-parametric test was used to test the association between each immune cell and vaccination type, separately for each type point. The permuted version of the Wilcoxon sing-rank test was used to test the distribution of measurements over time. To compare the changes between two time points for each cell type, fold changes in numbers of cells were used. The calculations carried out comprised the fit of linear mixed models, where the outcome was the log-transformed measurement and determinants were time points and vaccination type. The random effects part was specified as a random intercept. When measurements comprised zero's, the linear mixed model was fit on the original scale of the measurement and differences were reported instead of fold changes. Additionally, the longitudinal measurements of each immune cell were used to calculate the area under the curve of each participant's trajectory; the resulting measures were used in an association study in relationship to the vaccination type. The results of the tests were corrected for multiple testing with the Benjamini-Hochberg (BH) method applied separately to each sub-study. Reported differences and associations are deemed "significant" if the adjusted p-value amounts to less than 0.1, according to a false discovery rate of 10% (FDR < 10%).

The PCA was performed stratified per time point and at any time point by means of packages FactoMineR and factoextra in R. The variables were a priori standardized to have mean 0 and variance 1.

Summary statistics were performed by means of Graphpad Prism V7 (GraphPad, San Diego, CA, USA), while the hypothesis tests were performed in RStudio by means of the coin package (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/, version 1.3).

RESULTS

Study cohort characteristics

A total of twenty pre-menopausal healthy women between 23 and 44 years of age were included in this study. One participant dropped out at day 1 after the first vaccination and was replaced with a new participant. Women received three doses of either the bivalent vaccine (n=10) or the nonavalent vaccine (n=10) at 0, 2 and 6 months (Figure 1). The age of the participants who received the bivalent vaccine (32.7y.o, 95%CI 28.0-37.4) did not significantly differ from those receiving the nonavalent vaccine (31.6y.o., 95%CI 26.4-36.8).

Baseline and determination of optimal sampling time points after vaccination

As the immune status at the moment of vaccination can possibly influence the immune response, the cellular composition at day 0 as well as day 180 was used as baseline for immediate vaccine effects (day 0 before primary vaccination and day 180 before the third vaccination). At baseline and booster baseline, there were no significant differences in the total absolute numbers of cells in all immune subsets between the two cohorts (Mann-Whitney test, p.adjusted.value <0.3). However, the comparison of immune cell subsets between day 0 and day 180 did show significant differences, all p.adjusted.values<0.05. This shows that the median measurement of the participants at day 0 is significantly different for the median measurement of the cellular subsets at day 180, both before and after stratification for vaccine cohorts (Supplementary Table 2).

Preliminary experiments in the first five participants were designed to determine the most optimal post-vaccination timepoints for blood sampling to measure innate and adaptive immune cell numbers. The numbers of innate cells were evaluated at baseline (day 0), day 1, 2 and 3 post vaccination, as innate cells are expected to react directly upon vaccination [25]. Numbers of monocytes peaked at day 1 post vaccination in donors vaccinated with the bivalent vaccine, as was reflected by 2 out of 3 donors showing 1.3-fold or higher increase at day 1, when compared to baseline. The numbers of DCs increased at day 1 (mostly bivalent vaccine) and day 2 (mostly nonavalent vaccine; Supplementary Figure 1A). Changes in the numbers of T cells were minor and not consistent between donors. Of those limited changes, the response peaked as early as day 3, while in 3/5 others it peaked at day 7. The same was true for the total numbers of B cells, which increased in 2/5 donors from day 6 onwards. Plasma cell numbers peaked at day 7 post vaccination in 4/5 donors (Supplementary Figure 1B), thereby showing a clear expansion (Supplementary Figure 2). Therefore, to limit the number of sampling to a maximum of 10 and include relevant time points for each major population, we decided to include the timepoints day 0, 1, 3 and 7 (also used by others [26]) after primary vaccination for studying the cellular kinetics in the remaining 15 participants (7 in the bivalent- and 8 in the nonavalent cohort).

Strong alterations in circulating monocytes after vaccination

To study how innate cells respond to vaccination, ratios over baseline numbers of cells were calculated for all of the innate cell subsets analyzed at day 1 and day 3. A significant increase in median numbers of monocytes was only observed at day 1 in the bivalent vaccinated women (1.17 95%CI 1.003-1.37(Figure 2A). After the third vaccination, a significant increase in the numbers of monocytes was seen at day 181 in both cohorts, 1.27 95%CI 1.05-1.55 and 1.28 95%CI 1.05-1.57, respectively. Depending on their maturation stage, monocytes can be divided into three phenotypically distinct subsets ranging from classical (cMo) to intermediate (iMo) to non-classical (ncMo)[27]. These different monocyte subsets followed a different pattern in time after vaccination per vaccine type (Figure 2B). The samples in the bivalent cohort showed a significant early rise in numbers of iMo (1.72, 95%CI 1.17-2.53) at day 1. This rise in iMo was not significant after the third vaccination with the bivalent vaccine. For cMo a significant increase was observed at day 181, 1.29 95%CI 1.08-1.55. In the donors of the nonavalent cohort, only after the third vaccination, a rise in numbers of cMo was observed (1.26 95%CI 1.04-1.52) (Figure 2B). Statistically significant differences between the two cohorts were observed at day 1 for the absolute numbers of total monocytes, p.adjusted.value<0.01, iMo p.adjusted.value<0.01, and

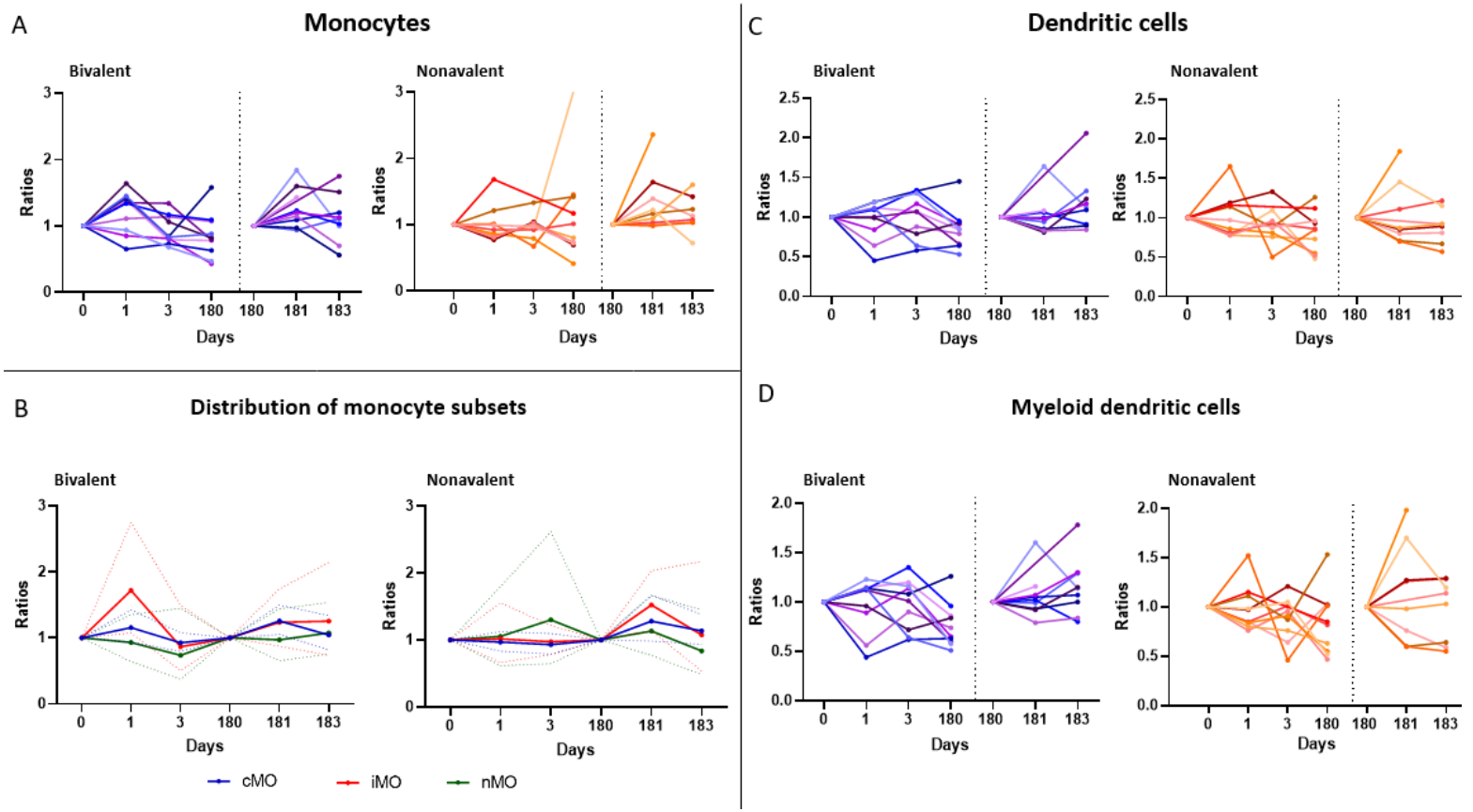


Figure 2 Monocytes (A), the distribution of monocytes (B), dendritic cells (C) and myeloid dendritic cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at days 0, 1, 3, 180, 181 and 183 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value. cMO= classical monocytes, iMO- intermediate monocytes and nMO are non-classical monocytes.

cMo p.adjusted.value<0.05. Overall, a higher number of circulating monocytes was observed upon bivalent vaccination than after nonavalent vaccination.

The total numbers of DCs (CD45+, CD33+, CD141+/-, HLA-DR+, FcERI+, CD14-, CD16-, CD303+/-) upon vaccination showed a heterogenous response between donors of both vaccine cohorts (Figure 2C). However, in some donors first an increase (day 1) and then a decrease (day 3) is observed, while in other donors this was reversed. In the nonavalent cohort the ratios of the DCs varied, but showed a non-significant trend at day 3 that suggest a decrease upon primary vaccination (0.86 95%CI 0.71-1.04). After the third vaccination this was similar (Figure 2C). The DCs can be subdivided into myeloid DCs (mDC) which mainly stimulate T cells, and plasmacytoid DCs which have a role in viral infections due to their interferon producing capacities [28]. We studied the ratios in numbers of mDCs (CD45+, CD33+, CD14- to dim+, HLA-DR+, FcERI+, CD16-CD141+/-) which showed a pattern similar to that of the total DCs (Figure 2D). Overall, no clear increase or decrease was observed over time in numbers of DCs in either cohort.

In the bivalent cohorts, median ratios of numbers of neutrophils increased at days 1 (1.46 95%CI 1.05-2.02) and 181 (1.42 95%CI 1.15-1.76) and returned to baseline or lower levels at days 3 and 183 (Supplementary Figure 3A). In the nonavalent cohort an increase was only observed after the third vaccination at day 181 (1.48 95%CI 1.18-1.85). Numbers of neutrophils differed significantly between the two cohorts at day 1 and 3 after primary vaccination, p.adjusted.value<0.01 and p.adjusted.value<0.05, respectively, being higher in the bivalent vaccinated cohorts.

Basophils are important in allergic response and eosinophils are involved in combating parasites [29], but are not likely to be involved in a response to vaccination. Basophils and eosinophils fluctuated independently of the primary vaccination with some differences between donors (Supplementary Figure 3BC). Surprisingly, after the third vaccination a significant increase for eosinophils was observed at day 183, with median ratios in numbers for bivalent and nonavalent donors of 1.44 95%CI 1.15-1.79 and 1.32 95%CI 1.04-1.68, respectively (Supplementary Figure 3C). Natural killer (NK) cells showed neither a significant increase or decrease at day 3 or day 7, depending on the donor, in both cohorts. However, after the third vaccination there was a clear peak at day 3 in 8 out of 10 donors of the bivalent cohort, this response occurred somewhat later in time (day 7) in the nonavalent cohort (Supplementary Figure 3D).

Heterogenous changes in T cell numbers after vaccination

CD4 T cell ratios of the bivalent cohort increased either at day 3 (2 donors) or day 7 (6 donors) post vaccination (up to 1.33 and 1.32 fold above baseline, respectively; Figure 3A), albeit no significant increase or decrease for median values was observed after primary and third vaccination. CD4 T cell ratios did not change after primary and third vaccination in the donors of the nonavalent cohort. The number of CD4 T follicular helper (TFH) cells, which help B cells in the formation of germinal centers, generally followed the kinetics of total CD4 T cells (Figure 3A). Overall changes for CD4 T cells and CD4 TFH cells were subtle, but homogenous between donors.

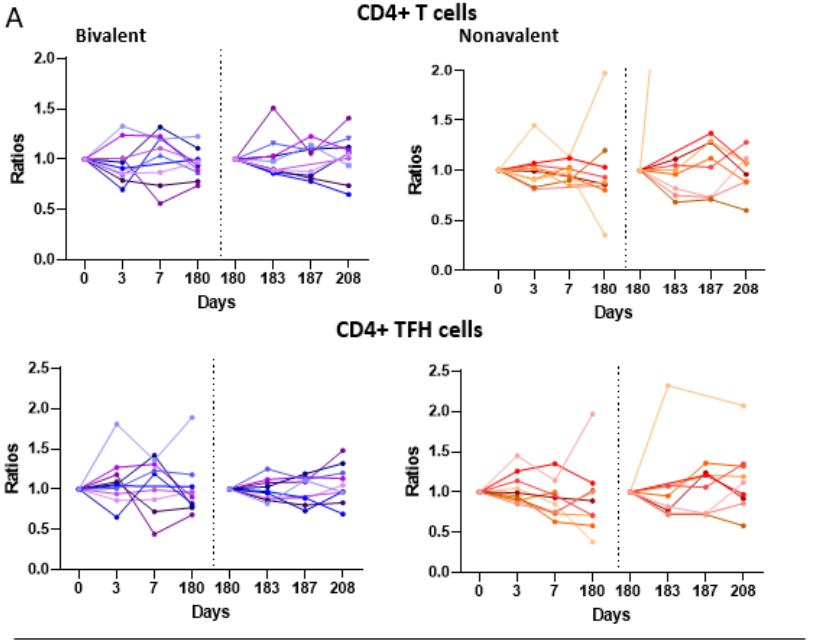
In general, the numbers of CD4 T-helper 1 (Th1) like cells (CD183+ CD194- CD196- CCR10-) decreased in the first week after primary vaccination in both the bivalent and the nonavalent cohort, with medians of 0.69 95%CI 0.56-0.86 and 0.79 95%CI 0.64-0.98 at day 3 and 7 respec-

tively and for nonavalent donors 0.82 95%CI 0.66-1.02 and 0.90 95%CI 0.72-1.11 at day 3 and 7, respectively (Figure 3B). After the third vaccination, the median ratios in numbers of CD4 Th1-like cells increased to a similar extent in both cohorts at day 3 but the change was only significant in the nonavalent cohort 1.69 95%CI 1.06-2.71. The numbers of CD4 Th2-like cells (CD183- CD194+ CD196- CCR10-) in both the bivalent and nonvalent cohort had a heterogenous response. The numbers of CD4 Th17-like cells (CD183- CD194+ CD196+ CCR10-) showed a significant decrease at day 7 in the bivalent cohort of 0.44 95%CI 0.20-0.97. In the nonavalent cohort, an inconsistent pattern of the response in the first week after primary vaccination was observed. After the third vaccination, no significant changes were observed in both the bivalent and nonavalent cohorts. In the bivalent cohort, median ratios of numbers of CD4 Th22-like cells (CD183- CD194+ CD196+ CCR10+) showed a decrease at day 7, 0.38 95%CI 0.15-0.99. After the third vaccination an increase was observed, despite not being significant, 2.06 95%CI 0.92-4.60 and 2.05 95%CI 0.89-4.72 at day 183 and 187, respectively. In the nonavalent cohort a heterogenous response was observed upon primary vaccination between the participants. After the third vaccination this was the same. In Supplementary Figure 4, CD4 T regulatory (Treg) cells, CD8 T cells and gamma delta T ($TCR_{\gamma\delta}$) cells are shown, these cells fluctuated independently of vaccination and showed a high variation between donors (Supplementary Figure 4BCD).
Long-term HPV16/18/31 and 45-specific IFN-γ producing cells were detectable in all donors at day 208. The bivalent cohort showed consistently higher numbers of specific IFN-γ producing cells compared to the nonavalent cohort, being significant upon HPV16 and HPV45 stimulation, p=0.029 and p=0.026, respectively (Figure 3C).

Plasma cell expansion and maturation after 7 days post primary vaccination

In both cohorts the ratio of plasma cells (PC) above baseline showed a strong increase at day 7 after primary vaccination, up to 7.65 fold and 34.23 fold for the bivalent and nonavalent cohort, respectively (Figure 4A). This increase was significant in the nonavalent cohort (3.01 95%CI 1.347-6.60) and almost significant in the bivalent cohort (2.15 95%CI 0.98-4.71). After the third vaccination there was a non-significant trend towards an increase at day 187 in both cohorts (Figure 4A). In Figure 4B and Supplementary Figure 5, changes in ratios of numbers of different subclasses (IgG, IgA, IgD and IgM) of plasma cells are presented. In both the bivalent and nonavalent cohort, a significant increase is seen at day 7 for IgG1 subclasses, 3.03 95%CI 1.11-8.31 and 5.80 95%CI 2.12-15.90, respectively. In the nonavalent cohort a significant increase is observed for IgG2 and IgM, 2.27 95%CI 1.19-4.30 and 5.20 95%CI 1.53-17.72, respectively. A significant decrease was observed for IgG4 for both the bivalent and nonavalent cohort, 0.02 95%CI -0.02-0.08 and 0.03 95%CI -0.01-0.06, respectively. After the third vaccination, significant increases are only observed in the bivalent cohort at day 187, in IgG1 (3.11 95%CI 1.14-8.49) and in IgG3 (10.68 95%CI 3.81-29.94). In the nonavalent cohort no significance increase was observed, but for IgG3 an increasing trend was observed (2.97 95%CI 1.00-8.82). Furthermore, plasma cells can be divided into maturation stages based on the expression of CD20 and CD138. Most immature plasma cells are CD20+CD138-, then they become CD20-CD138- and most mature plasma cells are CD20-CD138+. All these maturation stages can be measured in blood [30]. Total plasma cells showed an increase in maturation phenotype (CD20-CD138+) at days 7 and 187 (data not shown).

An increase in numbers of memory B cells was not observed in the first week after primary and the third vaccination in both cohorts (Figure 4A). HPV-specific memory B cells for type 16, 18,



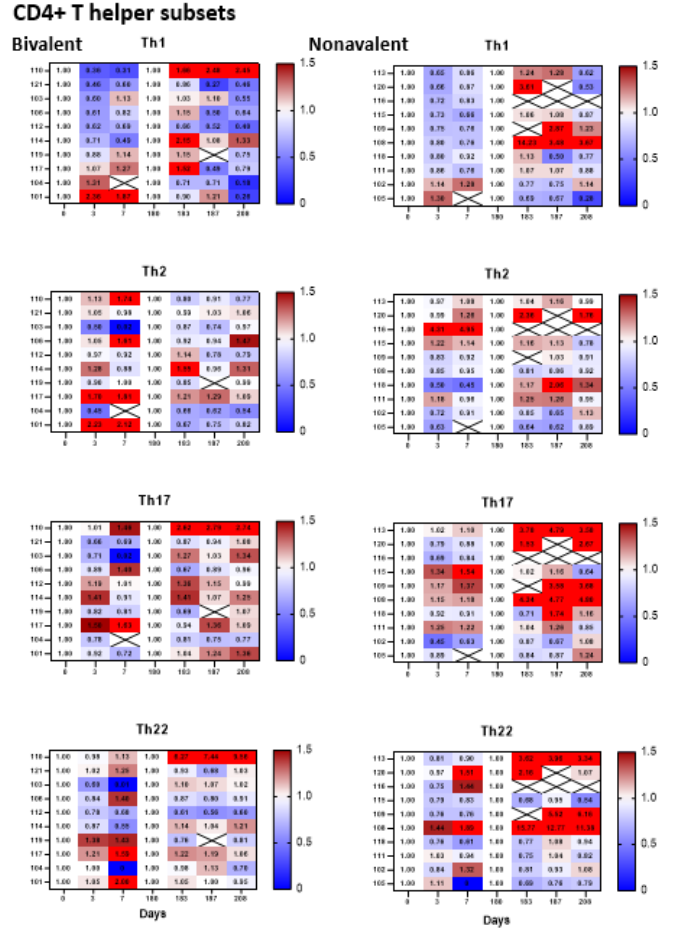


Figure 3 CD4+ and CD4+ TFH T cells (A) and CD4+ T helper subsets (B) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 3, 7, 180, 183, 187 and 208 post vaccination. Values >1.5 are depicted in bright red. HPV16/18/31/45 specific memory CD4+ T cells, X indicates non-reportable value at that timepoint (C) at day 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value. *p<0.05

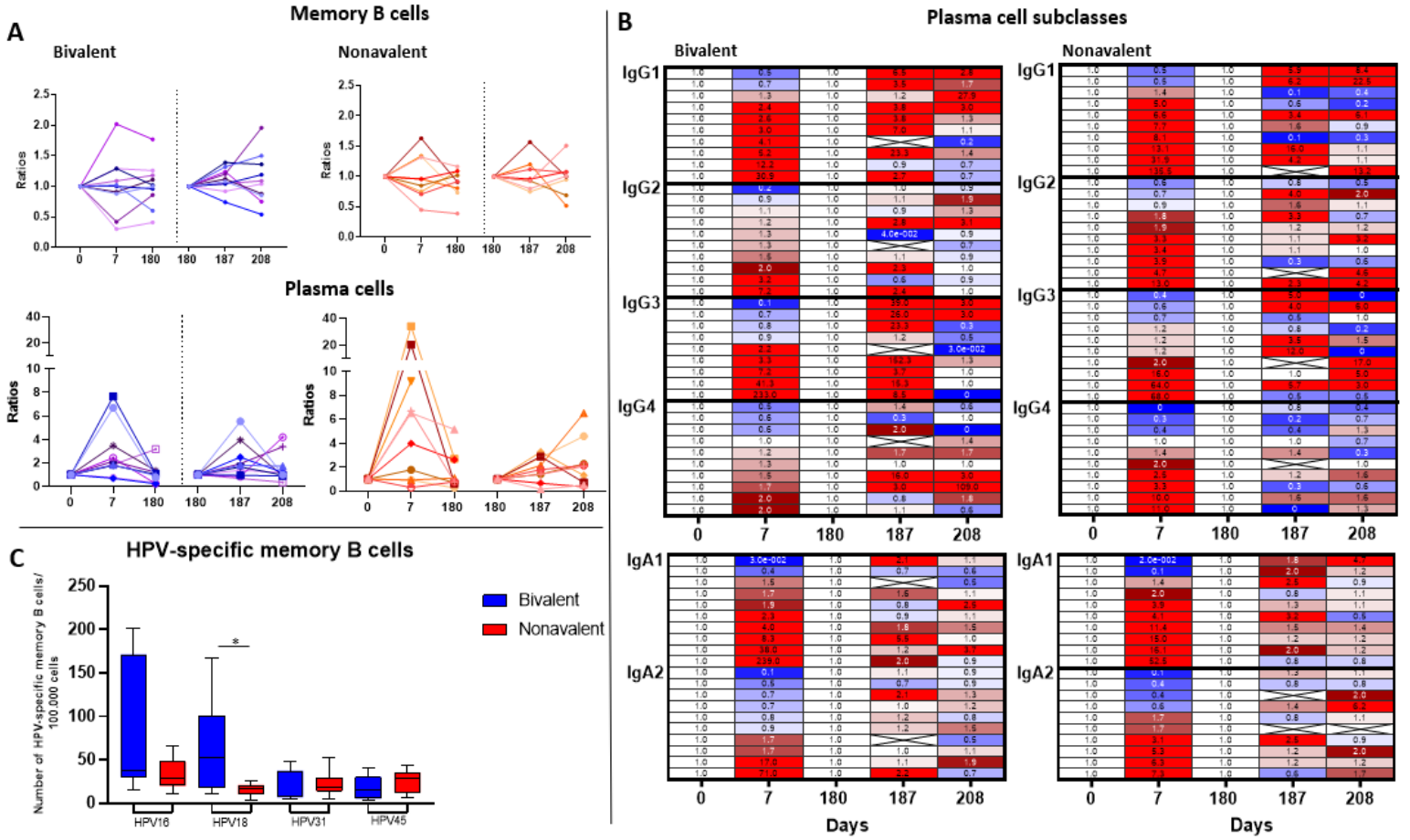


Figure 4 Memory B cells, plasma cells (A) and plasma cell distribution responses (B) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 7, 180, 187 and 208 post vaccination. Values >2.0 are depicted in bright red. HPV16/18/31/45 specific memory B cells, X indicates non-reportable value at that timepoint (C) at day 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.*p<0.05.

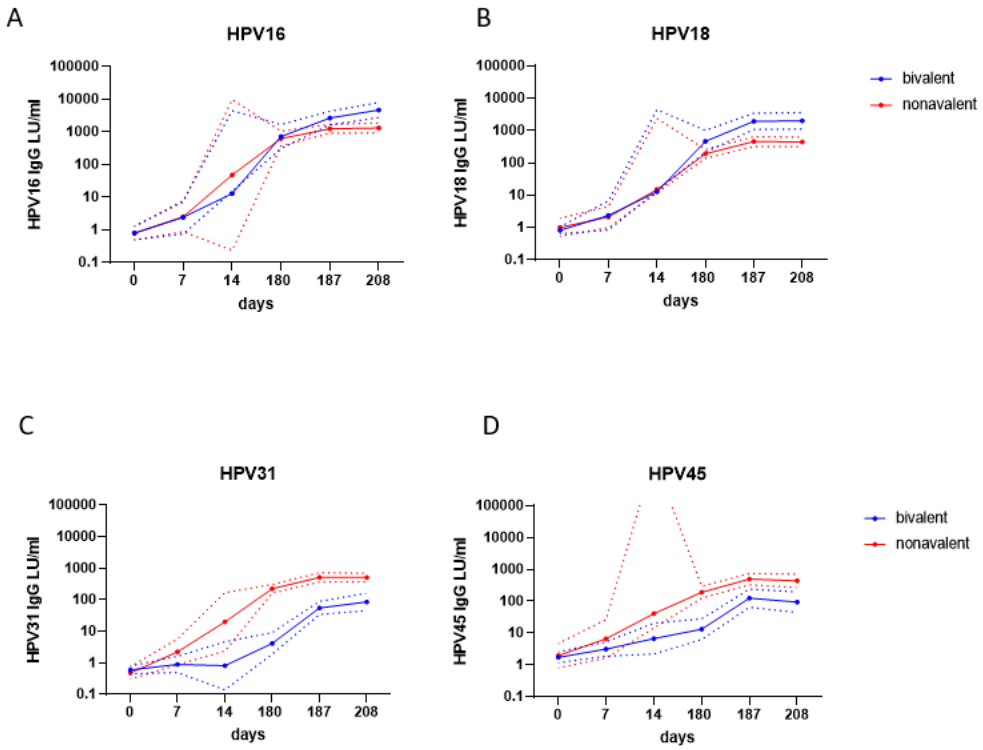


Figure 5 HPV 16/18/31/45 specific IgG antibodies IgG upon bivalent (blue) and nonavalent (red) vaccination at day 0, 7, 14, 180, 187 and 208 post vaccination. The line represents the GMC at each timepoint and the sashed lines represents the 95% confidence interval.

31 and 45 were all detectable at a month post third vaccination (day 208). The bivalent vaccinated donors showed significantly higher numbers of HPV18-specific memory B cells than those in the nonavalent cohort, p=0.035, and slightly higher but not significant for numbers of HPV16-memory B cells (Figure 4C).

High HPV-specific antibody responses in both vaccination cohorts

HPV-specific IgG antibodies were induced by both vaccines and their levels increased from day 7 up to day 187 after vaccination and seemed to plateau at day 208 (Figure 5). The GMC for HPV16 and 18-specific IgG levels was significantly higher in the bivalent cohort than the nonavalent cohort at day 208, p=0.0011 and p=0.0003, respectively (Figure 5AB). As HPV31 and HPV45 were not present in the bivalent vaccine, HPV31 and -45 IgG-specific antibody levels were significantly higher in the nonavalent cohort compared with the bivalent cohort, p<0.0001 and p=0.0015, respectively (Figure 5CD). HPV-specific IgA levels showed similar patterns as for IgG but did not show significant differences between the cohorts (Supplementary Figure 6). The most abundant HPV16,18,31,45 IgG subclass induced after both the bivalent and nonavalent vaccination was IgG1, followed by IgG3. Very small amounts of IgG2 and IgG4 were found. HPV16, 18 and 31 specific IgG1 distributions were significantly higher in nonavalent vaccinated women. HPV16, 18 and 31 specific IgG3 distributions were higher in nonavalent vaccinated women (Supplementary Table 3).

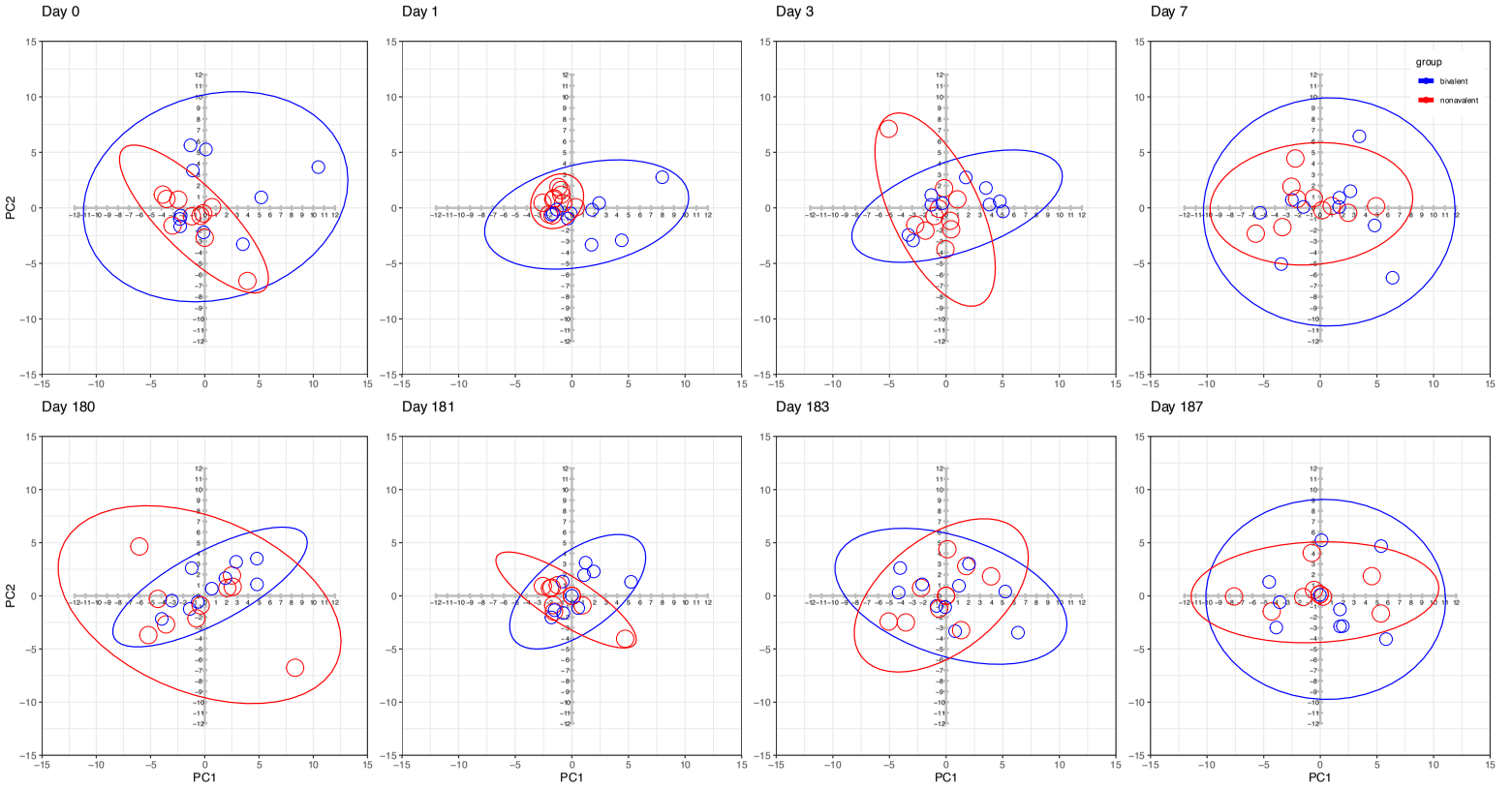


Figure 6 Visual representation of the principal component analysis where we describe the individuals by vaccination group, where group "bivalent vaccination" is denoted by 0 (blue) and group "nonavalent vaccination" is denoted by 1 (red) at days 0, 1, 3, 7, 180, `181, 183 and 187 post vaccination. The size of the eclipses visualizes the contribution of individuals to the first two principal components.

Individual responses post vaccination per donor in the major cell subsets in both vaccination cohorts

In Supplementary Figure 6 the absolute numbers of cells for the four major cell lineages (monocytes, B cells, plasma cells and T cells) over time are indicated per participant. The graphs are ordered based on the magnitude of plasma cell expansion. The donors showed a heterogenous response. In seven of the donors with a clear plasma cell expansion, no response in their numbers of monocytes was observed. In the remaining four donors with a less prominent plasma cells expansion an increase in the numbers of monocytes is observed. Six donors with a stable, or even slight decrease, in the numbers of plasma cells appear to have an increase in their numbers of monocytes. In the remaining two, no or a slight decrease in the numbers of monocytes is observed. Numbers of total B cells and CD4 T cells appear to remain similar to baseline levels overtime, and do not seem to be affected by vaccination.

Multidimensional comparisons of kinetics of different cell types

To study the long-term response, we looked for correlations between the IgG levels at day 208 with numbers of memory B- and T cells at day 208, CD4+ T cells and plasma cells at various timepoints, per HPV type, stratified per vaccine cohort. No significant correlations were found with HPV16-specific IgG levels at day 208 (Supplementary Table 4). We also studied the correlations between IgG subclasses and IgG subclass plasma cells at day 187, here also no significant correlations were found (data not shown).

By means of the PCA analysis, we determined at day 1 after primary and booster vaccination (days 181), the variation between all individuals was generally being explained by numbers of monocytes and mDCs, at days 3 and 183, this was equally explained by the numbers of innate cells and T cells. At day 7 the numbers of plasma cells explained most of the variance between individuals, while at day 187 this was explained by the numbers of CD4 T cells and B cells.

Also by means of PCA analysis, we determined potential differences between the two vaccine cohorts in the variation of induction of the various circulation numbers of cells during time after vaccination. We projected the input of all immune cell types at each time point onto 2 dimensions and analyzed the pairs of cells that contributed to differences in the phenotypes of cells between the vaccines. At day 1 the Wilcoxon-Mann-Whitney test of association showed evidence of differences in distributions of cells between the two vaccination groups, $p=0.04$. At this day only innate cellular subsets were measured, so this means that the vaccinations have a different effect on the expansion of innate cell subsets at day 1 after vaccination. At the other timepoints there was no evidence of differences in distributions of cellular subsets between the two groups (Figure 6).

DISCUSSION

In this study, we investigated the kinetics of various immune cell subsets in the circulation following primary and third vaccination with the bivalent and nonavalent HPV vaccines using in-depth immunophenotyping by means of state-of-the-art flow cytometry. These cellular kinetics were correlated to HPV-specific antibody levels and numbers of long-term memory B- and T cells and were compared between the bivalent and nonavalent HPV vaccines. The numbers of innate cells, especially that of monocytes, showed a clear expansion one day after vaccination in both cohorts and was significantly higher in the bivalent cohort than in the nonavalent cohort. In both cohorts, the numbers of plasma cells expanded in the first week after both primary and third vaccination, being significant only upon primary vaccination. In all other cellular subsets, a large heterogeneity in responses between the donors was observed. The HPV16 and 18-specific antibody levels and memory B and T cell responses were higher in the bivalent than the nonavalent cohort at one month after the third vaccination. This was opposite for HPV31 and -45 levels, which are not contain in the bivalent vaccine.

Innate cells, which form the first line of defense against infections, react quickly to vaccine antigens via pattern-recognition receptors like toll-like receptors (TLRs). The adjuvant AS04 is described to interact via monophosphoryl lipid A (MPL) with TLR4, which is frequently present on antigen presenting cells (APCs) [31, 32]. Innate cells, such as monocytes serve several functions within the immune system, most importantly phagocytosis, antigen presentation and cytokine secretion [33]. In this study, the numbers of innate cells have been determined at baseline and day 1 and day 3 after vaccination. Especially the numbers of monocytes, and their corresponding subsets, together with that of neutrophils, changed in the circulation at day 1 post vaccination. Monocytes and dendritic cells react upon vaccination as APCs and further migration to the secondary lymph nodes to be able to present the vaccine antigens, whereas the other innate cells studied have these capacities to a lesser extent [33]. Monocytes differentiate into cMo, iMo and nMo groups [34], in our study we found that absolute counts of iMo and cMo are higher in the bivalent cohort compared with the nonavalent cohort at the first day after vaccination. Which can be explained by the TLR4 agonist present in the adjuvants of the bivalent vaccine. Moreover, the PCA plots showed us that at day 1 the contribution of the monocytes of the bivalent vaccinated donors to the variance in the total observed response was greater than that of the nonavalent vaccinated donors. Although differences in magnitude of monocyte responses between cohorts are in line with the literature, we cannot exclude that part of these differences can be attributed to the differences in the kinetics of the immune response. Since changes in the innate compartment are very dynamic and the number of samples is limited, even a minor change in the response time may have a large effect on direct comparisons. Especially for iMo, that showed a clear peak in the bivalent cohort at day 1, this peak in numbers of iMo may reflect an effect of the vaccine in the activation of monocytes. We also observed higher numbers of neutrophils in the bivalent cohort than found in the nonavalent cohort within the first days after vaccination, suggesting that the nonavalent vaccine induces a more moderate innate immune response.

Dendritic cells (DC) are classical APCs and should become activated upon vaccination. However, DCs in blood are only found in an immature state and upon activation rapidly migrate to the lymph nodes, to infiltrate into the tissue at the site of administration [35]. We observed a

heterogenous response in numbers of circulating DCs directly after vaccination and in consequence we were unable to show clear kinetics in the DC response. Another study, making use of mass cytometry, did however show expansion of DCs upon vaccination [36], but an attenuated vaccine was used instead of a VLP-based viral vaccine and different timepoints (from day 5) were chosen. Mobilization of leukocytes into the circulation is highly likely to be specific for the type of vaccine, as also shown by the data presented here.

Following the initial wave of the innate cells, the adaptive part of the immune system becomes activated. The numbers of CD4 T cells showed an increase from day 3 up to day 7 after primary vaccination in the bivalent cohort, which was less pronounced in the nonavalent cohort. This is in line with the observed HPV-specific IFN-y responses. After the third vaccination no clear expansion was seen for both cohorts, similar to what was reported for antigen-specific T-cell upon booster vaccination with the hepatitis B vaccine [37]. Also, the individual plots show no clear effect of vaccination (bivalent or nonavalent) on numbers of CD4 T cells. CD4 TFH cells, which support the activation and differentiation of B cells into Ig-secreting plasma cells [38], showed a heterogenous response and were not found to be correlated to B cells in our study. The adjuvant present in the bivalent vaccine (AS04) has been shown to be capable of inducing a Th1 T-cell response in mice [14], whereas the adjuvant present in the quadrivalent and nonavalent vaccine mainly induced a Th2 T-cell response in animal models [39]. In contrast, human clinical data so far only show limited differences in vaccine-induced T cell (cytokine) profiles between the bivalent and quadrivalent vaccine, as assessed by expression of IL-2, IFN-y, and TNF-a [40], which is confirmed by our data. Long-term HPV-specific IFN-y producing cell levels, which we use as a measure for the HPV-specific T cell responses, were significantly higher for type 16 and 45 in the bivalent cohort when compared with the nonavalent cohort. This is in agreement with other studies [40, 41], that describe a trend towards higher IL-2 and TNF-α levels in bivalent vaccinated individuals than quadrivalent ones. So, although the aluminum adjuvanted vaccines (quadrivalent and nonavalent) also induces a Th1 response, this is higher in the AS04 adjuvanted vaccinated individuals. However, the precise underlying mechanism still needs to be unraveled and the relevance to protection .

Plasma cells, which are responsible for the production of antibodies, show a clear expansion at day 7 after primary vaccination with any of the two vaccines, which is also clear in the individual plots. Moreover, plasma cells also show to be the highest contributor to the variance in immune response observed at this timepoint in the PCA data. After the third vaccination this effect was diminished. It could be that we have missed the peak in plasma cells upon the third vaccination, since an expansion in plasma cells will be quicker due to already induced memory B cells. This was supported by a study showing a plasma cell expansion after the third dose of a rabies vaccination already at day 4 post booster vaccination [42], which was not included in our analysis.

Adjuvants, such as AS04, that activate TLRs are thought to induce antibody class switching [15]. In the bivalent cohort, an increase in specifically the subclasses IgG1, IgG2, IgG3 and IgA1 was observed. In the nonavalent cohort, IgG1, IgG2 and IgA1 showed the biggest increase. Remarkable was that IgG3 especially increased after the third vaccination compared with primary nonavalent vaccination. In contrast, our HPV-specific IgG subclass data show a significant higher contribution of IgG3 in bivalent vaccinated women than that in nonavalent vaccinated women.

Spearman correlation analysis did not show any correlations between IgG-subclass-specific plasma cells with HPV-specific IgG-subclass responses at day 187. This could either be explained by kinetics, as the antibodies induced by the plasma cells still need to be formed at day 187. Otherwise it could be that a part of the induced IgG-subclass specific plasma cells are not HPV-specific. In other studies, bivalent vaccinated women showed especially an IgG1 and IgG3 antibody profile [43], whereas quadrivalent, containing the same adjuvant as the nonavalent vaccine, showed high levels of IgG4 and IgA in addition to the IgG1 and IgG3 response [41, 44]. This, together with our results, suggests that the bivalent vaccine is better capable of inducing an IgG3 response. Since IgG3 is related to a potent pro-inflammatory response [45, 46], which could perhaps explain the higher immunogenicity of the bivalent vaccine compared to the nonavalent. HPV-specific memory B cells at one month after the third vaccination showed higher numbers for HPV16 and 18 in the bivalent cohort compared with the nonavalent cohort, which is in line with other studies comparing the bivalent and quadrivalent vaccine [5-7]. These studies also showed that the bivalent vaccine induce higher antibody levels for HPV16 and 18 than the quadrivalent vaccine [6, 8]. This is in line with our findings, where long-term HPV-specific IgG antibody levels for HPV16 and 18 were significantly higher in the bivalent cohort.

In individuals that received the bivalent vaccine, cross-reactivity and cross-protection against HPV types absent in the vaccine is observed and is mostly attributed to the AS04 adjuvant [7]. Although this is observed in many studies [6, 8, 9, 13], the mechanism explaining this is still lacking. Another additional explanation could lie in the difference in the structure of the L1 protein in the two different vaccines as besides the differences in adjuvants, also different L1 expression systems are being used for the synthesis of the HPV VLPs. The VLPs used in this study to measure HPV were made with the baculovirus expression system, resembling the VLPs of the bivalent vaccine. However, since others observed similar differences between bivalent and quadrivalent vaccinated individuals while making use of recombinant proteins [8], we do not expect that this would lead to any bias. The bivalent L1 proteins are produced using a baculovirus expression vector system and purified to be able to form VLPs. These VLPs consist of important conformation-dependent neutralizing epitopes, such as U4, V5 and J4, thereby closely resembling the native HPV virions [47, 48]. Also the shape of the VLPs produced in baculovirus was found to be more consistent [49], when compared to the VLPs produced in the yeast expression system [50]. So, presumably the adjuvant AS04 in the bivalent vaccine contributes to the high and a long-lasting immune response. Additionally, the conformation of the VLPs of the L1 produced protein in the yeast expression system might be less optimal for inducing cross-protective antibodies whereas those produced in baculovirus might have a better conformation that allows cross reactivity. Together with the potent innate response observed upon bivalent vaccination, this might cause the higher memory T cell responses that subsequently result in a plasma cell expansion and the corresponding antibody production.

The immune subsets, measured using the EuroFlow tubes, are not antigen-specific, providing us only with information about changes in blood cell numbers of multiple (>250) immune cell subsets upon vaccination. It allows us to monitor in-depth innate, B cell and T cell immune responses at different time points after vaccination in a highly standardized and reproducible manner. However, a limitation of this study was the high heterogeneity observed between do-

nors, together with the low sample size, therefore no clear cell responses, except for plasma cells, could be identified.

Both vaccines induced detectable B and T cell responses, although HPV-specific numbers were higher in bivalent vaccinated women, and lead to high levels of antibodies. Further research in this area could be performed by looking at HPV-specific B and T cells after vaccination. The B cell repertoire [51] could give us insight in the type of memory B cells that are formed upon either bivalent or nonavalent vaccination, possibly giving us an explanation of the observed cross-protection in bivalent vaccinated women.

The HPV field is just beginning to understand the potential implications of innate and/or adaptive immune signatures and adjuvant effects on the generation of effective adaptive immune responses.

To get an insight into the impact of the cellular response on the efficacy of the currently used vaccines may be of importance. This is needed for a more extensive insight in how the innate immune response is linked to long term immunity.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1 Inclusion and exclusion criteria of the EVI study

Inclusion criteria	Exclusion criteria
• Seronegative for high risk-HPV vaccine types (16,18,31,33,45,52,58); • Female; • Normal general health; • Pre-menopausal; • Willing to receive HPV vaccination; • Provision of written informed consent; • Willing to adhere to the protocol and be available during the study period.	• Present evidence of serious disease(s) within the last 3 months before inclusion requiring immunosuppressive or immune modulating medical treatment, such as systemic corticosteroids, that might interfere with the results of the study; • Chronic infection; • Known or suspected immune deficiency; • History of any neurologic disorder, including epilepsy; • Previous administration of serum products (including immunoglobulins) within 6 months before vaccination and blood sampling; • Known or suspected allergy to any of the vaccine components (by medical history); • Previous vaccination with any HPV vaccine; • Pregnancy; • Participation in another vaccination/ medicine study.

Supplementary Table 2 Corrected P values of the associations between the repeated measurements at day 0 and day 180, by the Wilcoxon signed-rank test

Cell subset	P.adjusted. value
Bcell	0
ImmatureBcell	0
NAIVECD5Bcell	0
NAIVEBcell	0
MemBcell	0
MemBcellIgMD	0
MemBcellIgG1	0
MemBcellIgG2	0
MemBcellIgG4	0
MemBcellIgA1	0
MemBcellIgD	0
PCIgM	0
PCIgG1	0
PCIgG2	0
PCIgG3	0
PCIgG4	0
PCIgA2	0
PCIgD	0
Tcells	0
CD4	0
CD4TFH	0
CD4TFHnaive	0
CD4TFHTh1	0
CD4TFHTh2	0
CD4TFHTh17	0
CD4Treg	0
CD4naive	0
CD4Th1	0
CD4Th2	0
CD4Th17	0
CD4Th22	0
CD4Th1.17	0
TCRgd	0
CD8Tcells	0
Nkcells	0
Leukocytes	0
Eosinophils	0
Neutrophils	0
Basophils	0
Monocytes	0
cMO	0
iMO	0
nMO	0
DC	0
mDC	0
HPV16IgG	5,54E-05
MemBcellIgA2	6,51E-05
PC	0,000138
PCIgA1	0,000916
CD4TFHTh1.17	0,020145
MemBcellIgG3	0,04081

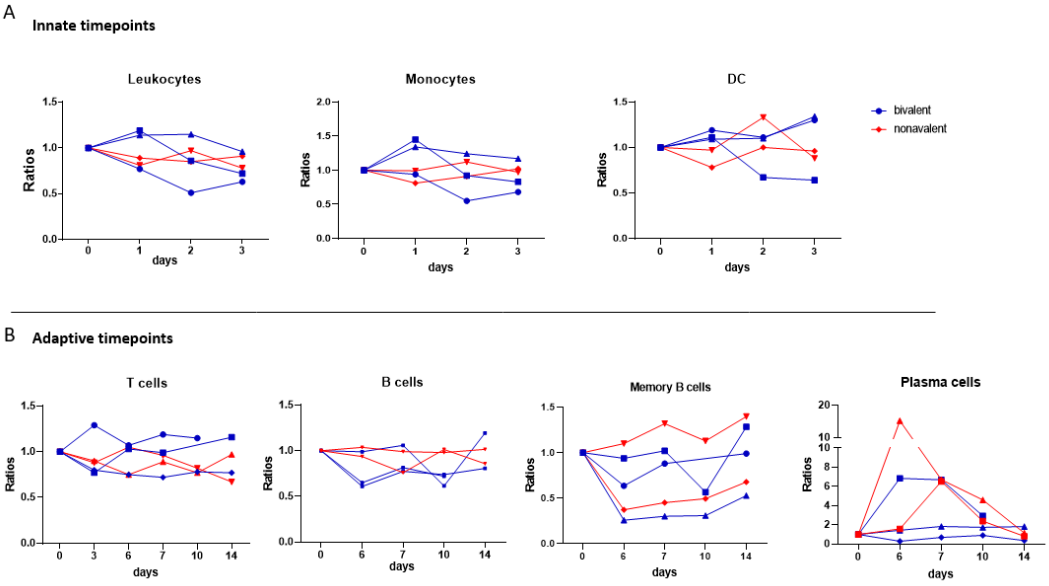
Supplementary Table 3 HPV16,18, 31 and 45-specicifc IgG-subclass percentages of the total HPV-specific IgG production

HPV16								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
Mean	53.92*	74.95*	0.11*	0.23*	45.9*	24.67*	0.07*	0.15*
95% CI	39.3-68.5	67.2-82.8	0.04-0.17	0.15-0.31	31.3-60.5	16.9-32.5	0.03-0.11	0.12-0.18
HPV18								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
Mean	49.7*	86.9*	0.15	0.17	50.0*	12.8*	0.13	0.16
95% CI	35.4-64.1	81.2-92.7	0.07-0.24	0.11-22	35.6-64.4	7.0-18.5	0.05-0.22	0.14-0.19
HPV31								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
mean	20.4*	79.9*	0.26	0.21	79.3*	19.7*	0.02*	0.17*
95% CI	13.6-27.2	72.7-87.1	0.0-0.62	0.13-0.29	72.4-86.2	12.5-26.6	0.0-0.06	0.15-0.19
HPV45								
	IgG1		**IgG2**		**IgG3**		**IgG4**	
	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent	bivalent	nonavalent
mean	68.6	76.7	0.16	0.28	31.2	22.6	0.07	0.46
95% CI	56.4-80.8	61.9-91.4	0.0-0.34	0.0-0.66	19.1-43.2	8.8-36.4	0.0-0.14	0.0-1.10

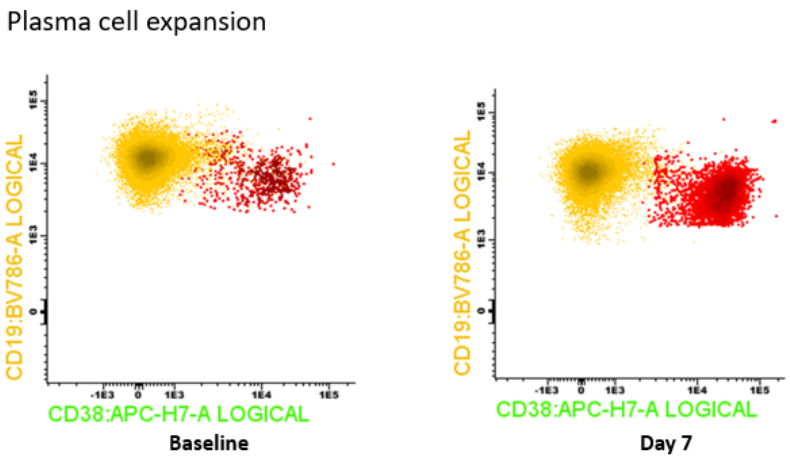
*$p<0.05$

Supplementary Table 4 Correlation between the long-term HPV16-antibody levels with different cellular subsets at various timepoints, stratified per vaccine cohort.

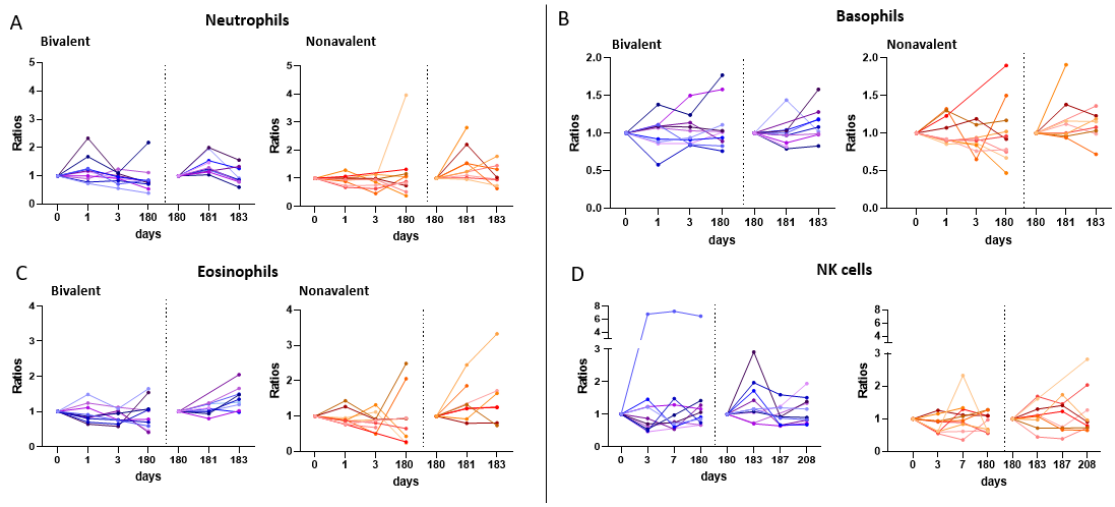
Cellular subset	Bivalent cohort		Nonavalent	
	R^2	P-value	R^2	P-value
Monocytes day 0	0.218	0.173	0.031	0.626
Monocytes day 1	0.065	0.476	0.009	0.789
Monocytes day 3	0.146	0.277	0.178	0.258
Monocytes day 180	0.149	0.270	0.025	0.662
Monocytes day 181	0.002	0.909	0.068	0.534
Monocytes day 183	0.017	0.742	0.046	0.611
DC day 0	0.162	0.249	0.095	0.388
DC day 1	0.068	0.467	0.010	0.787
DC day 3	0.041	0.575	0.054	0.549
DC day 180	0.003	0.889	0.052	0.526
DC day 181	0.036	0.626	0.001	0.931
DC day 183	0.007	0.827	0.013	0.786
CD4+ T cell day 0	0.041	0.573	0.002	0.894
CD4+ T cell day 3	0.002	0.911	0.002	0.902
CD4+ T cell day 7	0.002	0.911	0.027	0.650
CD4+ T cell day 180	0.008	0.811	0.001	0.947
CD4+ T cell day 183	0.022	0.680	0.073	0.518
CD4+ T cell day 187	0.001	0.999	0.001	0.941
CD4+ T cell day 208	0.054	0.520	0.004	0.866
HPV16 mem CD4+ T cell	0.002	0.906	0.056	0.540
B cell day 0	0.003	0.887	0.041	0.576
B cell day 7	0.050	0.535	0.096	0.383
B cell day 180	0.108	0.354	0.022	0.706
B cell day 187	2.62x10-5	0.990	0.050	0.598
B cell day 280	0.032	0.621	0.019	0.722
Plasma cell day 0	0.040	0.578	0.019	0.705
Plasma cell day 7	0.071	0.457	0.210	0.188
Plasma cell day 180	0.043	0.567	0.003	0.894
Plasma cell day 187	0.159	0.287	0.419	0.083
Plasma cell day 280	0.113	0.342	0.098	0.413
HPV16 mem B cell	0.045	0.555	0.304	0.124



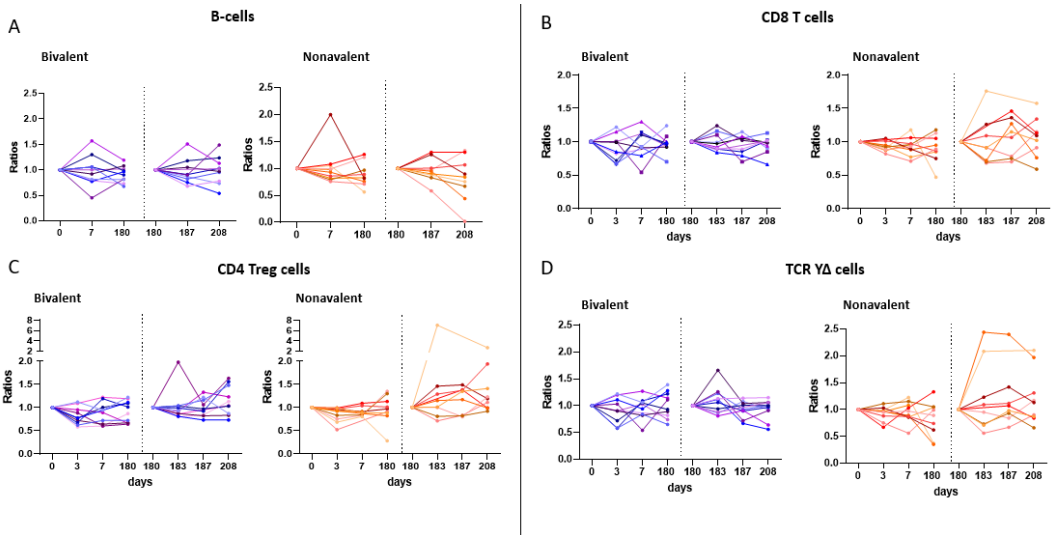
Supplementary Figure 1 Time finding results of the innate timepoints; leukocytes, monocytes and DCs at day 0, 1, 2 and 3 (A) and of the adaptive timepoints; T cells, B cells, memory B cells and Plasma cells at day 0, 3, 5, 6, 7, 10 and 14 after vaccination of either the bivalent (blue) or nonavalent (red) vaccine in the first five participants. Fluctuations of cells are presented as ratio compared to baseline value.



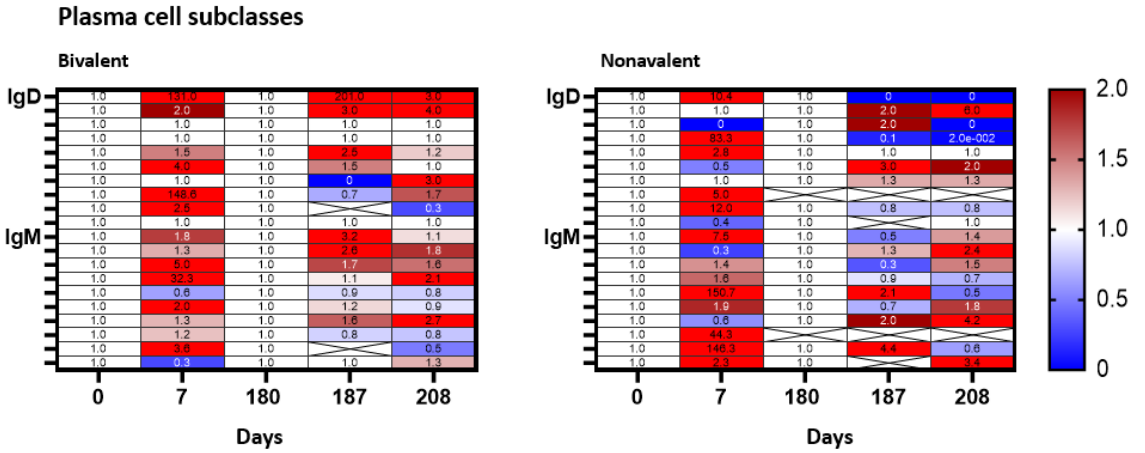
Supplementary Figure 2 Example of a plasma cell expansion from baseline to day 7 post vaccination of a bivalent donor. Plasma cells are depicted in red, memory B cells are depicted in yellow.



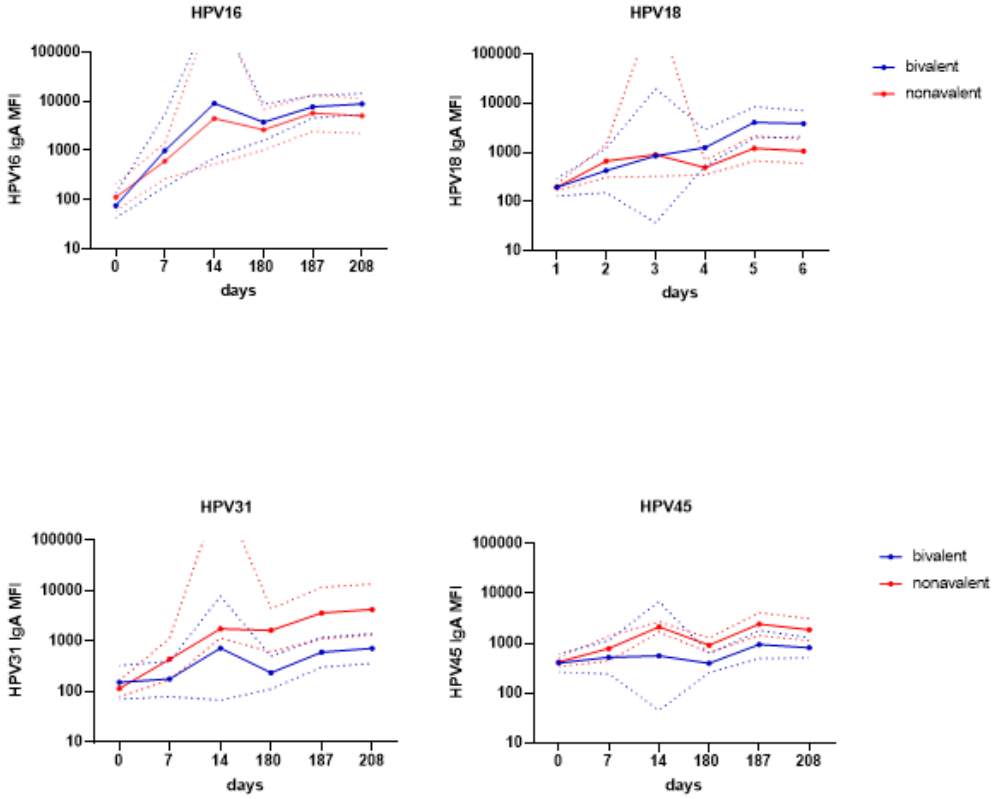
Supplementary Figure 3 Neutrophils (A), basophils (B), eosinophils (C) and NK cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 1, 3, 7 180, 181, 183 and 187 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.



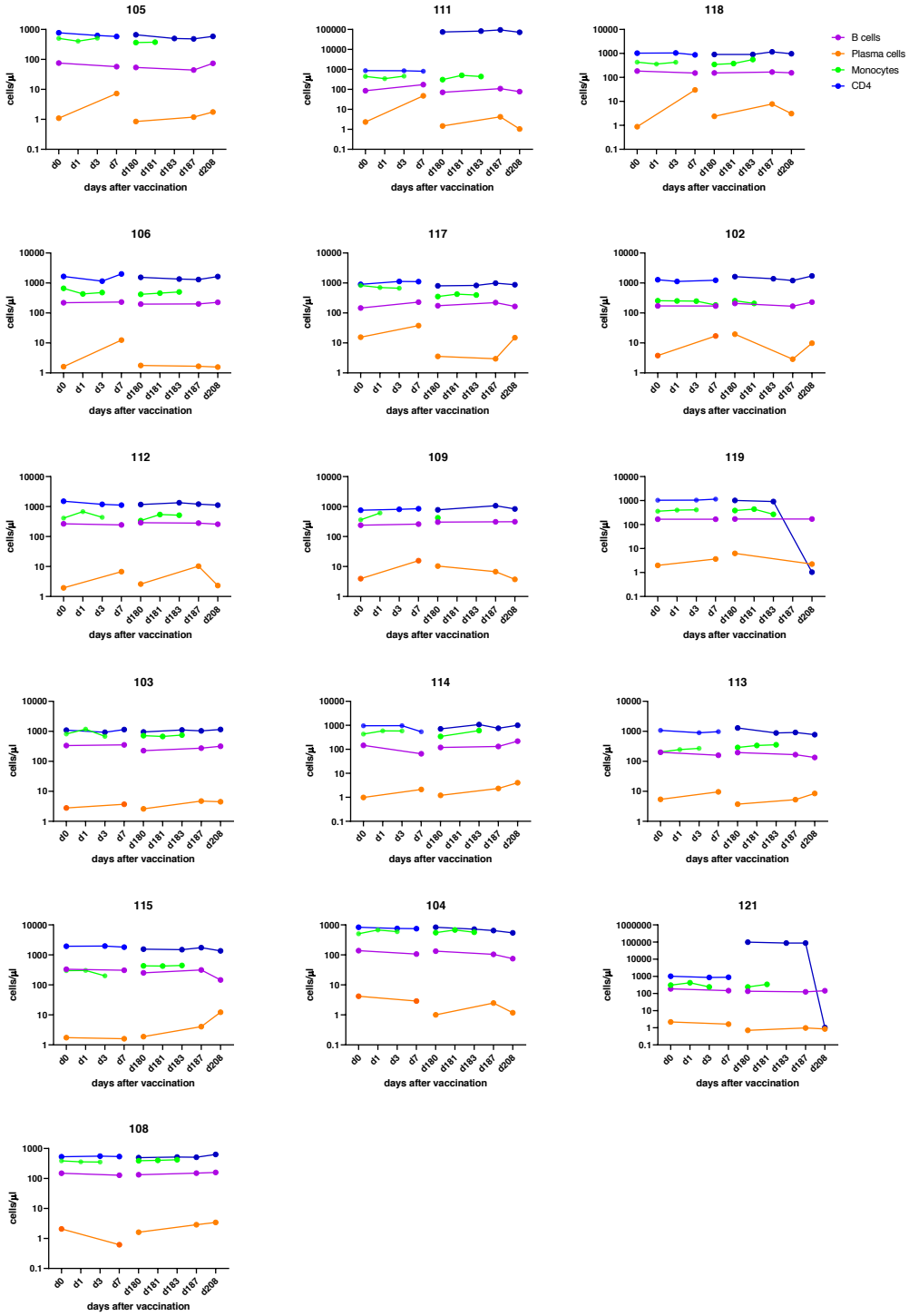
Supplementary Figure 4 B cells (A), CD8 T cells (B), CD4 Treg cells (C) and TCR YΔ cells (D) upon bivalent (purple-blue) and nonavalent (orange-red) vaccination at day 0, 3, 7 180, 183, 187 and 208 post vaccination. Fluctuations of cells are presented as ratio compared to baseline value.



Supplementary Figure 5 Plasma cell subclasses upon bivalent and nonavalent vaccination at day 0, 7, 180, 187 and 208 post vaccination. Values >2.0 are depicted in bright red.



Supplementary Figure 6 HPV 16/18/31/45 specific IgA antibodies IgG upon bivalent (blue) and nonavalent (red) vaccination at day 0, 7, 14, 180, 187 and 208 post vaccination.



Supplementary Figure 7 Absolute numbers of cells of the four most important cell lineages, monocytes (green), B-cells (purple), plasma cells (plasma cells) and CD4 T cells (blue), during time postvaccination per donor.