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Seasonal and Nonseasonal Longitudinal Variation of Immune Function

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Different components of the immune response show large variability between individuals, but they also vary within the same individual because of host and environmental factors. In this study, we report an extensive analysis of the immune characteristics of 56 individuals over four timepoints in 1 single year as part of the Human Functional Genomics Project. We characterized 102 cell subsets using flow cytometry; quantified production of eight cytokines and two chemokines in response to 20 metabolic, bacterial, fungal, and viral stimuli; and measured circulating markers of inflammation. Taking advantage of the longitudinal sampling, both seasonal and nonseasonal sources of variability were studied. The circulating markers of inflammation IL-18, IL-18 binding protein, and resistin displayed clear seasonal variability, whereas the strongest effect was observed for α-1 antitrypsin. Cytokine production capacity also showed strong seasonal changes, especially after stimulation with the influenza virus, *Borrelia burgdorferi*, and *Escherichia coli*. Furthermore, we observed moderate seasonality effects on immune cell counts, especially in several $CD4^+/CD8^+$ T cell subpopulations. Age of the volunteers was an important factor influencing IFN-γ and IL-22 production, which matched the strong impact of age on several T cell subsets. Finally, on average, genetics accounted for almost 50% of the interindividual variance not already explained by age, sex, and body mass index, although this varies strongly for different parameters. In conclusion, seasonality is an important environmental factor that influences immune responses, in addition to specific genetic and nongenetic host factors, and this may well explain the seasonal variation in the incidence and severity of immune-mediated diseases. *The Journal of Immunology*, **2021, 207: 696–708.**

Over our lifetime, we are exposed to countless pathogens and other environmental immune triggers, and each of these encounters impacts our immune system to varying degrees. Some of these effects are long term, for instance immunological memory after responding to a specific pathogen, whereas others are short-lived, like an acute infection by a pathogen. There are individual baseline differences in our immune status that are independent of environmental influences, due to host factors like age, sex, or genetic background. Recent research has shown that both these general host factors and the environment play important roles in determining the state of our immune system (1–12). As a consequence, there are large differences in most immune parameters even within a healthy population, subsequently influencing susceptibility to infection, allergies, and autoimmunity.

Interindividual variation generally refers to variation between individuals, whereas intraindividual variation refers to variation within an individual over time. Some immune characteristics will be mostly independent of the environment, with variation being predominantly interindividual, whereas others will be more susceptible to input from external factors, resulting in large intraindividual variation.

Interindividual variation of the immune system has been a topic of great interest in the last few years (1–12). However, a comprehensive overview of the contribution of both the interindividual and intraindividual variation for a wide range of immunological parameters is still lacking. Intraindividual variation of the immune system in particular has been poorly studied, mostly because of a lack of longitudinal sampling or because only a few immune parameters

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Abbreviations used in this article: AAT, α-1 antitrypsin; BMI, body mass index; EM, effector memory; 500FG, 500 Functional Genomics; hsCRP, high-sensitive CRP; IL-18BP, IL-18 binding protein; LOESS, locally estimated scatterplot smoothing; 56P, 56-Periodic; Treg, regulatory T cell.

were assessed (2, 7–9, 11, 13, 14). Among external factors, the impact of seasonality on the immune responses has been shown to be important (1, 6, 12). However, because often just a single timepoint is measured, this effect is almost always neglected. This has hampered our ability to evaluate which immune characteristics are relatively stable and which are prone to variation over time, either seasonal or nonseasonal.

In the current study, we comprehensively quantified the percentage of variance that is explained by interindividual and intraindividual variation for a wide range of immune parameters. Specifically, the design of this cohort enables a detailed analysis of the seasonal influences on these parameters. In this study, we report an extensive analysis of the immune characteristics of 56 individuals over four timepoints in 1 single y. We termed this cohort the 56-Periodic (56P) cohort, which is part of the Human Functional Genomics Project (15). Participants were selected as a subset of a larger cohort termed the 500 Functional Genomics (500FG) cohort (12), which was recruited 2 y earlier, also allowing longer-term stability assessments (Fig. 1A). We characterized 102 circulating cell subsets using high-resolution flow cytometry, quantified the production of eight cytokines and two chemokines in response to twenty stimuli in vitro, and measured circulating markers of inflammation (Fig. 1B). Taking advantage of the longitudinal sampling, we studied in detail the interindividual and intraindividual variation of a large set of immune parameters and investigated seasonal influences. We report which immune parameters are tightly regulated by host factors such as sex, age (Fig. 1C), body mass index (BMI) (Fig. 1D), and genetic background and which immune parameters are strongly influenced by environmental factors such as season.

Materials and Methods

Experimental methods

56P cohort. Participants for the 56P cohort were selected as a subset of a larger cohort termed the 500FG cohort (described below) (12), which was recruited 2 y earlier, also allowing longer-term stability assessments. Fifty-six volunteers were included between February 2016 and February 2017 at the Radboud University Medical Center, Nijmegen, the Netherlands, all volunteers are of Western European origin. Immune parameters were assessed at four different timepoints, spaced roughly 3 mo apart, within 1 y (Fig. 1A). Out of the 56 volunteers, 3 volunteers missed a single timepoint. A range of parameters were measured, including circulating markers of inflammation, immunophenotyping of cell (sub)populations, and in vitro cytokine and chemokine production after stimulation with different pathogens and compounds (Fig. 1B, Supplemental Table I). Participants had a mean age of 39.6 ± 17.4, 60.7% were male, and the average BMI was 24.0 ± 3.1 (Fig. 1A, 1C, 1D).

500FG cohort. The 500FG cohort consists of 534 healthy individuals of Western European genetic background and is part of the Human Functional Genomics Project (10, 12, 16). Inclusion of volunteers took place between August 2013 and December 2014 at the Radboud University Medical Center, Nijmegen, the Netherlands. Forty-five individuals were excluded in the final analysis after examining the answers of their questionnaire and genetic profile, leaving 489 individuals. This study was approved by the Ethical Committee of Radboud University Medical Center, Nijmegen, the Netherlands (NL42561.091.12, 2012/550). Inclusion of volunteers and experiments were conducted according to the principles expressed in the Declaration of Helsinki. All volunteers gave written informed consent before any material was taken.

Definition of interindividual and intraindividual variation. In this study, four longitudinal measurements were performed over 1 single y, with roughly one measurement in each season. Therefore, we define interindividual variation as the variation between individuals over a time span of 1 y (i.e., the stable immunological differences between individuals over 1 y). Intraindividual variation is defined as the variation within individuals over the same time span, caused by changing environmental influences and altered host factors.

Circulating markers of inflammation. Circulating concentrations of resistin, leptin, adiponectin, CRP, IL-18, and IL-18 binding protein (IL-18BP) and α-1 antitrypsin (AAT) were measured in EDTA plasma using R&D Systems DuoSet ELISA kits following the manufacturer's protocol.

PBMC stimulation experiments. Isolation of PBMCs was performed as described in Oosting et al. (17). Briefly, PBMCs were isolated within 6 h after blood drawing by density gradient centrifugation of PBS diluted blood (1:1) over Ficoll-Paque. They were washed two times with PBS subsequently resuspended in Dutch modified RPMI 1640 medium (Invitrogen) supplemented with 2 mM GlutaMAX, 50 μg/ml gentamicin (Centrafarm), and 1 mM pyruvate (Life Technologies). Stimulations were performed with 5×10^5 cells per well in round-bottom, 96-well plates (Greiner) at 37°C and 5% CO_2 for either 24 h (short term) or 7 d (long term) in the presence of 10% human serum. After incubation, supernatants were collected and stored at −20°C until ELISA measurements were performed. The different stimuli that were used are shown in Supplemental Table I.

Cytokine measurements. Cytokine concentrations in PBMC cell culture supernatants were measured using commercially available ELISA kits following the protocols supplied by the manufacturer. As the induction of distinct sets of cytokines can strongly depend on the activation and differentiation of different subsets of immune cells present in the PBMC

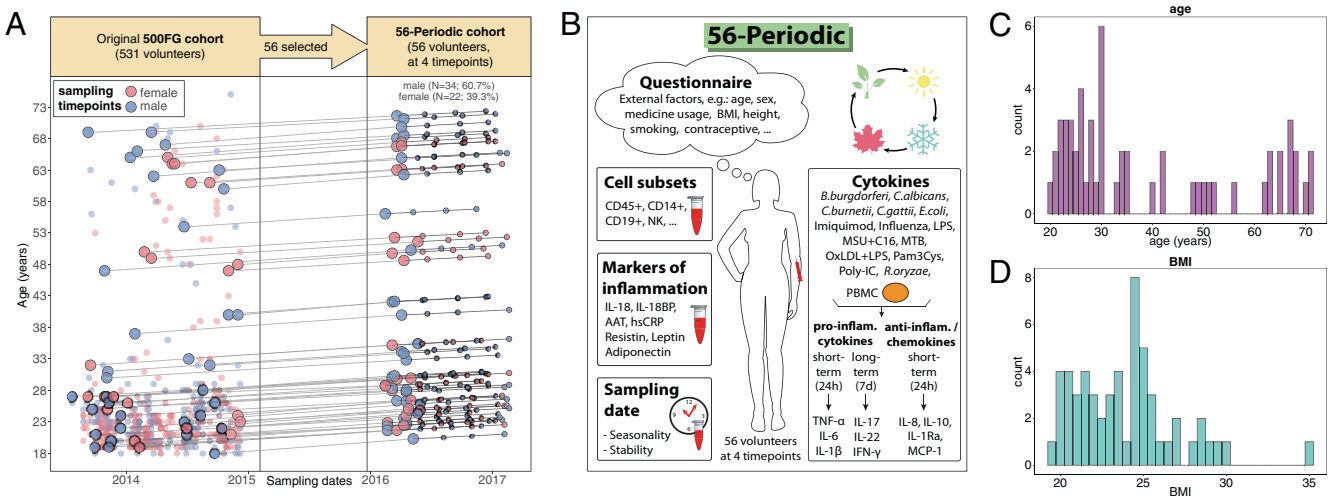

FIGURE 1. Characteristics of the 56P cohort and its relationship to the 500FG cohort. (**A**) Sampling overview, in which on the left, the original 500FG cohort is displayed with each dot indicating a single individual. Fifty-six of these individuals were sampled four more times as part of the 56P cohort, and these are indicated by larger dots. These are connected with the four timepoints of the 56P cohort on the right side, in which the first timepoint is marked with a larger dot. (**B**) Overview of the measurements performed for the 56P cohort. The measurements include circulating markers of inflammation, immune cell phenotypes, and cytokine production capacity upon stimulation. (**C**) Distribution of age of the 56P cohort. (**D**) Distribution of BMI of the 56P cohort.

mixture, requiring varying amounts of time, we stimulated PBMCs for two different timespans. We measured the cytokines IL-1β, TNF-α, IL-6, IL-1Ra, and IL-10 after 24 h of stimulation, which we refer to as short-term cytokines. Of these, IL-1β, TNF-α, and IL-6 are considered proinflammatory, and IL-1Ra and IL-10 are considered anti-inflammatory. The chemokines MCP-1 and IL-8 were also measured after 24 h of stimulation. In contrast, we quantified the proinflammatory cytokines IL-17, IL-22, and IFN-γ after 7 d of stimulation, and we will therefore refer to these as long-term cytokines. Cytokine quantification was performed using ELISA kits from R&D Systems (IL-1β, TNF-α, IL-6, IL-1Ra, IL-8, MCP-1, IL-17, and IL-22) or Sanquin (IL-10 and IFN-γ).

Flow cytometry. The complete flow cytometry procedure has been described before (1). In brief, blood was collected in 10-ml BD Vacutainer spray-coated K2EDTA tubes. Fresh peripheral blood cells were counted using a Coulter Ac-T diff cell counter (Beckman Coulter, Brea, CA). Cells were immediately processed and analyzed 2–3 h after sample collection on a three-laser Navios flow cytometer (Beckman Coulter). Supplemental Table XI lists the fluorochrome conjugate Abs (and their respective clones) purchased from Beckman Coulter (Marseille, France), Becton Dickinson (San Jose, CA), eBioscience (Vienna, Austria), or BioLegend (San Diego, CA) that were used in the five complementary Ab panels. All reagents were titrated and tested before they were used in the current study.

Flow cytometry data were analyzed using Kaluza software version 1.5a (Beckman Coulter). For flow cytometry data analysis, a manual hierarchical gating strategy was performed by two independent technical specialists to prevent gating errors. The gating strategy of panels 1–4 has been described before (1); for panel 5 (chemokine receptor panel), the gating strategy is presented in Supplemental Fig. 7. Analyzed data were stored batchwise per 20 samples each. The statistics were exported batchwise for further analysis.

Flow cytometry consistency. To check the across panel variation, we analyzed four cell subsets that were redundant between panel 1 and panel 2. The average correlation between the same markers in different panels was 0.90 (range 0.86–0.96). To ensure that results were consistent between panels, we calculated the inter- and intraindividual variation and compared these between panels (Supplemental Table XII). Overall, the markers between panels give very similar results, indicating that variation in the measurements and gating between panels likely does not influence the results and conclusions significantly.

Differential hematology analyzer. Platelets, basophils, eosinophils, RBCs, and hemoglobin and hematocrit levels in whole blood samples were measured on an automated hematology analyzer (XN-450, Sysmex system; Sysmex, Kobe, Japan).

CMV serology. CMV serology was measured using the CMV IgG ELISA from GenWay Biotech (catalog number GWB-BQK12C), following manufacturer specifications.

Statistical analysis

ANOVA. Using linear regression models and ANOVA, we quantified what portion of the variance is explained by interindividual variation and by intraindividual variation for each immune parameter. To calculate the percentages of explained variance, we used a type I (sequential) ANOVA, as implemented in the function anova from the stats package in the R programming language.

Specifically, the following general formula was used: immuneParamOfInterest ~ age + sex + BMI + individuals + season; here, immuneParamOfInterest is the immune parameter of interest. Age, sex, and BMI make up the first part of the interindividual variation, and the term individuals makes up the remainder of the interindividual variation. Individuals contain unique identifiers for each individual, which are dummy 0,1 coded internally by the anova function. Finally, season makes up the seasonal part of the intraindividual variation. The residuals make up the remainder of the intraindividual variation.

Seasonality analysis was performed similar to ter Horst et al. (12). In short, we used a linear combination of a sine and cosine term with the same frequency. This allows for the formation of a sine wave with any phase of that particular frequency. We used a sine and cosine wave with a period of 365 d, which allows us to determine the percentage of variance explained by any seasonal sine like pattern.

Significance of different influencing factors. To determine the significance of seasonality, we calculated if a model with a seasonal term was a significantly better fit to the data than one without a seasonal term using ANOVA. For the significance of age, sex, and BMI, we calculated the median values for each parameter over all timepoints and used linear regression to estimate significance. Specifically, we used the lm function from the stats package and the summary function in the R programming language. Taking the median does reduce the power of the tests for age, sex, and BMI compared with the test for seasonality. The p values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate multiple testing correction (18) per data type (e.g., all cell counts, all short-term cytokine production capacity, and all circulating markers of inflammation) for all factors of interest simultaneously (age, sex, BMI, and season). To phrase it differently, all combinations of variables of one data type with all factors of interest were corrected for simultaneously. The p.adjust function in the R programming language was used to perform the correction. The seasonality effect is filtered twice: first, it needs to be significant after multiple testing correction/multiplicity adjustment similar to the other features, and in the second stage, we filter for 2.5% explained variance. The reason for this second threshold is that there are several significant seasonal effects with a small percentage of explained variance. These were not considered to be biologically significant, and after evaluating the results, a threshold of 2.5% explained variance was set based on the consensus between several biologists.

Data correction. To correct for batch effects in the cytokine production capacity data, the batches were regressed out using linear regression. Also, the cell count data showed some slight drift over time, this was corrected using a linear term over time in a linear regression.

Data pretreatment. Analysis was performed using raw and/or log-transformed data and also using inverse rank-transformed data. For many parameters results were comparable, unless data deviated significantly from normality, generally because of one or more significantly higher/lower values. In these cases, the extreme values dominated the results. We therefore chose to report the results based on the inverse rank-based transformed data. Because we were interested in comparing between different cytokines and cell types, this provided the most reliable results. This does mean that the percentages of explained variance are in the space of transformed values and care has to be taken in interpreting the quantitative values.

Technical variation in cytokine production capacity. Changes in intraindividual variation could potentially be caused by technical issues, with less-potent or lower-dosage stimuli approaching the limits of detection, leading to increased relative noise and, thereby, potentially artificially increasing intraindividual variation. We therefore checked for each cytokine if there were correlations between median production capacity and intraindividual variation, considering each stimulus as a single data point. As shown in Supplemental Fig. 6A, no correlations were observed. To increase power, we standardized the median production capacity values per cytokine and performed the same analysis for each class of cytokines combined (short term, long term, short-term anti-inflammatory, and chemokines), and this also showed that there was no relationship between the potency of the stimulus and the intraindividual and interindividual variation. This means that the differences in interindividual and intraindividual variation we observe do not appear to be due to differences in the strength of stimuli.

Technical variation and cell abundance. Some general cell types are very abundant, whereas some further-differentiated cell types (lower in the treelike schema) have much lower numbers. We wondered if there was a relationship between the abundance of cell subtypes and their interindividual variation because high-frequency cells might be more reliably quantified. There was no relationship, with common and more-differentiated cell types showing on average the same intraindividual and interindividual variation (Supplemental Fig. 6B).

Percentage of variance explained by CMV. CMV seropositive status was only measured at a single timepoint (timepoint 2) and available for 52 out of 56 participants. We assumed that CMV serology results of individuals did not change over the course of the study. Also, given missing data for four individuals, the calculations for CMV serology status were performed separately from the analysis for the other parameters (age, sex, seasons, etc.). The same analysis was performed as described in the "*ANOVA*" and "*Significance* of *different influencing factors*" sections, including all other parameters and, additionally, adding binary CMV serology as an independent variable. However, the calculation was now performed for 52 instead of 56 individuals. Only the results for CMV are reported in this study; however, multiple testing correction was performed over all parameters considered to be a part of the inter- and intraindividual variation.

Plotting of seasonality. For the visualization of the seasonality, for each individual, the median value over all four timepoints was subtracted from all four timepoints. This way, we are removing (most of) the interindividual variation, leaving just the intraindividual variation. The fitted line and error range were plotted using the locally estimated scatterplot smoothing (LOESS) method of the geom_smooth function of the ggplot2 package and, again, is purely for visualization. These data manipulations are purely done for visualization and are independent of the statistical analyses described above.

Associations between cell counts/percentages and cytokines. To investigate the potential origin of the cytokines measured in whole blood and after stimulation of PBMCs, we calculated the associations between 1) cell percentages in PBMC as measured with a differential hematology analyzer and

cytokine production capacity measured in PBMC after 24 h (short term) and 7 d of stimulation (long term); and 2) cell counts as measured with flow cytometry and circulating makers of inflammation.

We constructed linear models using one variable from one data type (circulating markers of inflammation and cytokine production) as a dependent variable and one variable from another data type (cell percentages and cell counts) as an independent variable, correcting for age, sex, and BMI. We tried this using all data (with all four timepoints for an individual) and using the median value over all four timepoints. The rationale for the latter was that using all four timepoints could result in false associations because of correlated repetitive measurements. We see similar effects in both, but to prevent looking at biased results, we will only present the results based on median values in this study (Supplemental Table X). Given the number of associations we are evaluating and the limited number of samples when using the median values, only a few of the associations pass multiple testing correction. We therefore opted to include the p values not corrected for multiple testing to better show the patterns we observe.

Results

Interindividual and intraindividual variation

We and others have previously shown that sex and especially age have strong effects on many immune parameters and that BMI is strongly related to circulating markers of inflammation (1, 4, 7, 9, 11, 12). These factors are all considered part of the interindividual variation because sex is a stable property, and the age and the BMI of participants did not change significantly during the 1-y time span of our study, relative to the overall variation of these parameters. Seasonality is part of the intraindividual variation, and we and others previously showed season significantly influences many immune parameters (1, 6, 12). The remaining interindividual and intraindividual variation, not explained by age, sex, BMI and season, was quantified, and we classified this as other interindividual and other intraindividual variation.

Stability of circulating inflammatory markers

The concentrations of several serum markers of inflammation were determined, specifically the following: AAT, IL-18, IL-18BP, high-sensitive CRP (hsCRP), resistin, leptin, and adiponectin. The contributions of interindividual and intraindividual variance to the total variation are displayed in Fig. 2A (see also, Supplemental Table II). Leptin and adiponectin showed relatively small intraindividual variation (i.e., they are relatively stable over time), and they are strongly dependent on sex and BMI (Fig. 2A, 2B). hsCRP and especially AAT showed relatively large intraindividual variation (Fig. 2A, 2C). Given the seasonal nature of several infectious diseases and autoimmune diseases, it might be expected that some biomarkers of inflammation show seasonality. AAT showed a very strong seasonal signal, with a peak of circulating AAT concentrations in winter (Fig. 2A, 2D). Circulating IL-18, IL-18P, and resistin concentrations were moderately impacted by seasonality, peaking during winter (Fig. 2A, 2E). In line with previous observations, IL-18BP showed age and sex dependence (12), whereas IL-18 and hsCRP are associated with high BMI.

Stability of short-term cytokine production capacity

We previously showed that cytokine production capacity of human primary PBMCs is strongly influenced by sex and seasonality (12). In this study, we measured the capacity of PBMCs to produce IL-1β, IL-6, and TNF-α after stimulation for 24 h, which we will refer to as short-term cytokines, with 20 different metabolic, bacterial,

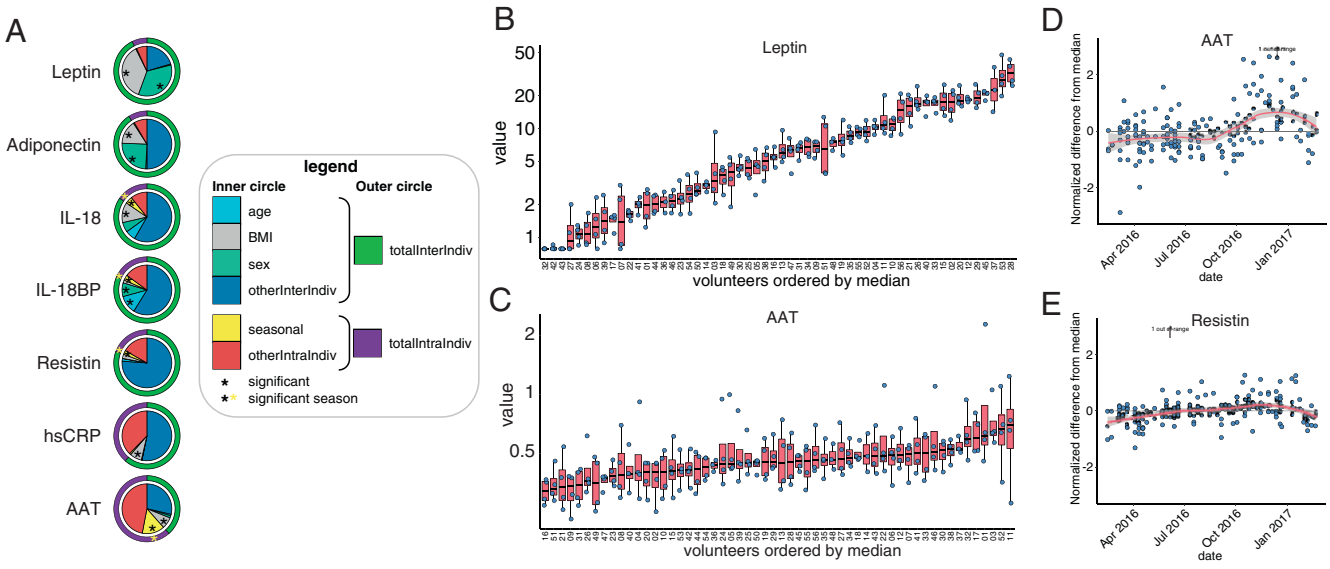

FIGURE 2. (**A**) Percentage of the total variation explained by interindividual and intraindividual variation for different inflammatory markers. The markers are ordered in increasing levels of intraindividual variation (top to bottom). The outer pie chart shows the total interindividual variation in green and the total intraindividual variation in purple. The inner pie chart shows the relative contributions of the different factors contributing to the interindividual variation and intraindividual variation. Interindividual variation is displayed using shades of blue, green, and gray and split into the effects of age, sex, BMI, and remaining (other) interindividual variation. Intraindividual variation is split into the effects of season (yellow) and the remaining (other) intraindividual variation (red). The factors that were significant after multiple testing (false discovery rate [FDR] corrected, $p < 0.05$) and explained at least 2.5% of the variance, which are marked with an asterisk. For variables with a significant seasonal component, and extra yellow asterisk is added for clarity. (**B**) Plot of the four leptin measurements for each volunteer, with the 56 volunteers ordered by their median value. Boxplots are plotted around the points as a way of visualizing the spread. (**C**) Similar to (B), but for AAT. (**D**) Plot showing the seasonal variation of AAT. In this plot for each individual, the median value over all four timepoints was subtracted from each of the four timepoints. This way, we are removing (most of) the interindividual variation, leaving just the intraindividual variation. This strategy was applied just for the visualization of the seasonality, and the statistical analyses are described in more detail in *Materials and Methods*. The fitted line and error range were plotted using the LOESS method of the geom_smooth function of the ggplot2 package and, again, is purely for visualization. (**E**) Similar plot to (D), but now for resistin.

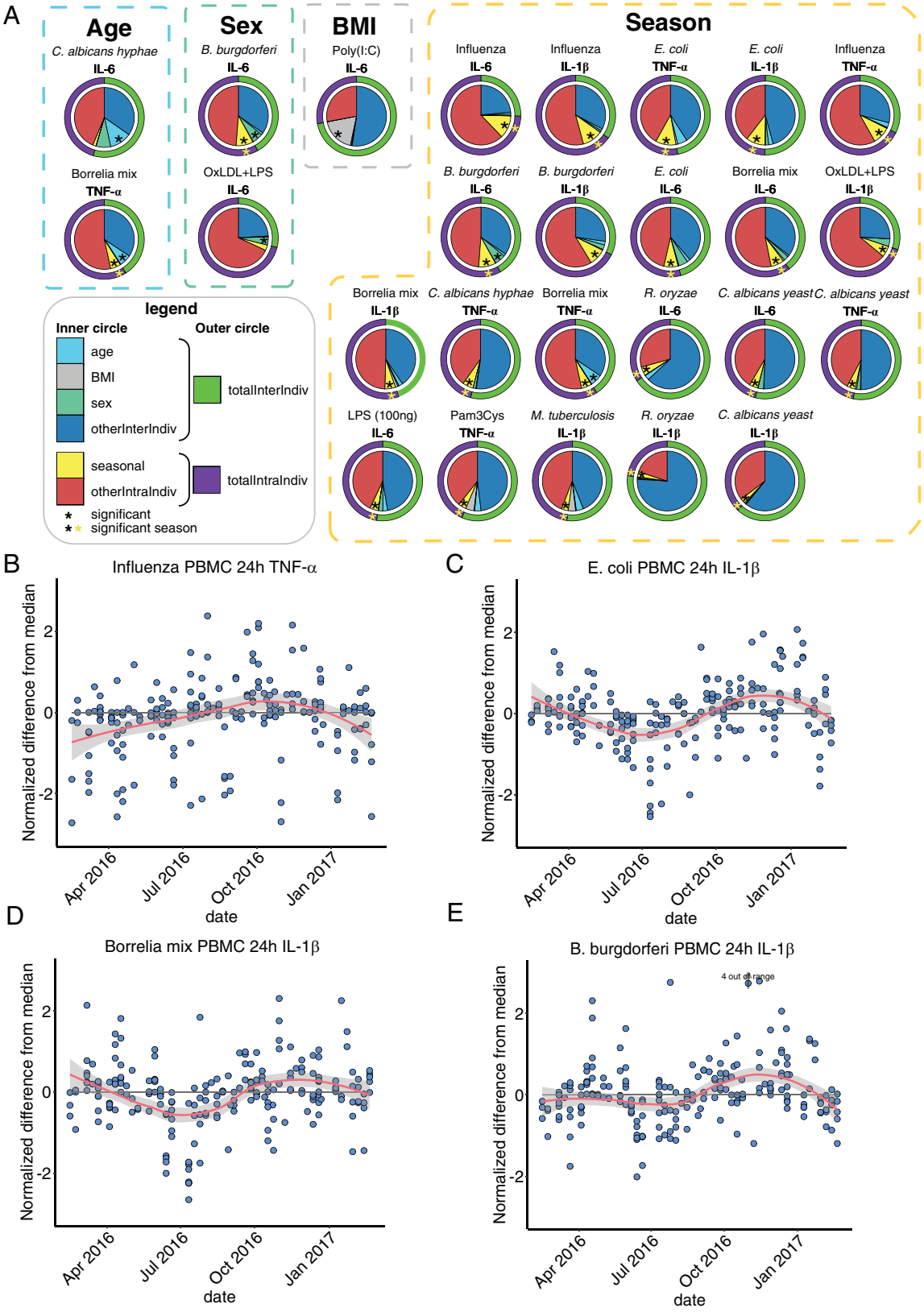

FIGURE 3. (**A**) Interindividual and intraindividual variation in monocyte cytokine production capacity after stimulation of PBMCs. Only cytokine–stimulus pairs that showed significant association with age, sex, BMI, and season are shown. The pie charts are shown in a similar format as in Fig. 2A. (**B**) Example seasonality plot for TNF-α production after influenza stimulation. In this plot for each individual, the median value over all four timepoints was subtracted from all four timepoints. This way, we are removing (most of) the interindividual variation, leaving just the intraindividual variation. This strategy was applied just for the visualization of the seasonality, and the statistical analyses are described in more detail in *Materials and Methods*. The fitted line and error range were plotted using the LOESS method of the geom_smooth function of the ggplot2 package and, again, is purely *(Figure legend continues)*

fungal, and viral stimuli and TLR ligands (Supplemental Table I). We observed large differences in inter- and intraindividual variation in cytokine production induced by the various stimuli. The variation between production of different cytokines for a single stimulus is much smaller, with IL-1β, IL-6, and TNF-α production showing very similar patterns (Supplemental Fig. 1, Supplemental Table III). *Rhizopus oryzae* evoked the most consistent cytokine responses over time, with more than 75% of the differences in the cohort being explained by interindividual (rather than intraindividual) variation for both IL-1β and TNF-α (Supplemental Fig. 1A, 1B). Still, seasonality had a small, but consistent, effect on cytokine production after stimulation with *R. oryzae*. Contrastingly, stimuli such as oxidized LDL + LPS, influenza, and *B. burgdorferi* resulted in large changes in cytokine production over time, with interindividual variation explaining only a little over 25% of the total variation.

Fig. 3A displays the cytokines and stimuli that are significantly associated with age, sex, BMI, and seasonality. Overall, the strongest effects were seen for seasonality, especially after stimulation with influenza, *Escherichia coli*, and *Borrelia burgdorferi*. Although we confirm our previous observation that cytokine production after stimulation with influenza is seasonal (12), we observed a slight shift in the peak of cytokine production (from August in 2014 to October in 2016) (Fig. 3B, Supplemental Table III). In addition, seasonality in response to stimulation with *B. burgdorferi* and a mix of *Borrelia* strains was observed, with lower cytokine production in July (Fig. 3A, 3D, 3E). Furthermore, strong seasonal effects in short-term cytokines were detected in response to *E. coli* stimulation, with a seasonal pattern similar to the one we observed for *B. burgdorferi* (Fig. 3A, 3C). Finally, age, sex, and BMI showed significant effects for only a limited number of cytokine–stimuli combinations (Fig. 3A).

Stability of long-term cytokines

As the production of several cytokines requires activation and differentiation of specific immune cells in the PBMC mixture, we additionally measured the long-term cytokines IL-22, IL-17, and IFN-γ after stimulation for 7 d. The production of these long-term cytokines showed relatively small differences in intraindividual variation between different stimuli (Supplemental Fig. 2A, Supplemental Table IV). The larger differences appear between cytokines; IFN-γ and IL-22 displayed a larger percentage of interindividual variation compared with IL-17. This may be explained by the strong effects of age on IFN-γ and IL-22 production (Fig. 4A, Supplemental Fig. 2) (12) Long-term cytokines are not influenced by seasonality as strongly as short-term cytokines. Nevertheless, we detected small, yet significant, effects of season on IFN-γ production after several stimuli (Fig. 4A); IFN-γ production is generally low in July, with the exception of stimulation with the TLR7/8 ligand imiquimod, showing an inverse pattern with a peak around July (Fig. 4B–E).

Stability of the anti-inflammatory cytokines IL -10 and IL-1Ra and the chemokines MCP-1 and IL-8

In addition to proinflammatory cytokines, we assessed the production of the short-term anti-inflammatory cytokines IL-10 and IL-1Ra and of the chemokines MCP-1 and IL-8 after 24 h of stimulation with LPS, Pam3Cys, *Candida albicans*, and *Staphylococcus aureus*. Production of IL-1Ra generally had slightly higher interindividual variation compared with production of IL-10 (Supplemental Fig. 2B, Supplemental Table V). For both IL-10 and IL-1Ra production, Pam3Cys stimulation resulted in the highest interindividual variation (Supplemental Fig. 2B). Similar to the anti-inflammatory cytokines, Pam3Cys is also the stimulus that resulted in the highest interindividual variation for chemokine production (Supplemental Fig. 2C, Supplemental Table VI). For the anti-inflammatory cytokine response, the strongest seasonality was observed for IL-10 production after *S. aureus* stimulation (Fig. 4F, 4H, 4I). Additionally, MCP-1 production in response to Pam3Cys stimulation showed a seasonal pattern (Fig. 4G, 4J), peaking at the end of August. IL-8 production after *C. albicans* stimulation showed an inverse seasonal pattern, with a decreased production around August (Fig. 4G, 4K).

Immune cell counts

Cell counts for 102 different cell (sub)populations were quantified at the four timepoints for all 56 individuals of the 56P cohort. The percentage of variance explained by interindividual and intraindividual variance is displayed in Fig. 5. The figure shows the cell types with significant contributions for age, sex, BMI, or season. An overview for all cell subtypes is displayed in Supplemental Fig. 3, in which they are displayed in a hierarchical treelike structure, starting with total leukocytes at the top and going down into progressively smaller cell subsets (see also, Supplemental Table VII). Some of the larger cell subsets, like the total number of immune cells in the circulation, lymphocytes, neutrophils, and monocytes, tended to have a relatively large percentage of intraindividual variation, meaning they varied substantially between the four timepoints. In contrast, some of the smaller daughter subsets of the main lymphocyte cell class had relatively low intraindividual variation (Supplemental Fig. 3); especially $CD3^+CD56^+$ T cells and NK cells were relatively stable over time (Fig. 5C).

There were several T cell subsets with great stability over time. For instance, various $CCR7^-CCR5^+$ T cells had relatively large interindividual variation, and the same holds true for $CD45RA^+CD27^-$ central memory T cells (Supplemental Fig. 3). Although not the focus of the current studies, these cells are of significant interest in the HIV infection field. As Fig. 5B shows, $CD8^+$ T cells showed changes in inter- and intraindividual variation as they mature. Four stages of maturation are shown: naive cells ($CD45RA^+$ and $CD27^+$), central memory cells ($CD45RA^-$ and $CD27^+$), effector memory (EM) cells ($CD45RA^-$ and $CD27^-$), and effector cells ($CD45RA^+$ and $CD27^-$). Naive $CD8^+$ T cell counts were age dependent and significantly higher in younger individuals. However, as they developed to central memory $CD8^+$ T cells, this age dependence disappeared. As these cells matured to EM and effector $CD8^+$ T cells, the interindividual variation rose to over 90% (i.e., their numbers became extremely stable over time). Within the $CD4^+$ T cells, we also observed an increase in interindividual variation as they matured to effector cells, although this was much less pronounced (Supplemental Fig. 3, Supplemental Table VII).

Besides naive $CD8^+$ T cells numbers, several $CD8^+$ T cell subset numbers decreased with age, in line with our previous findings (1), whereas several $CD4^+$ T cell subset numbers increased with age (Fig. 5A, Supplemental Fig. 3). Out of the $CD4^+$ T cells, the $CCR6^+$ memory regulatory T cells (Tregs) showed the strongest increases with age. Seasonality had a clear effect on cell counts, although generally not as strong as for cytokine production capacity. $CD4^+CD25^{high}$ Tregs were most strongly influenced by seasonality, showing an increase around October (Fig. 5D). Overall, mainly $CD4^+$ and $CD8^+$ T cells subsets showed seasonal changes,

for visualization. (**C**) Similar to (B), but now for *E. coli* stimulation. (**D**) Example plot showing seasonality of IL-1β production after stimulation of PBMCs with a mix of *Borrelia* bacteria. (**E**) Similar to (D), but now for *B. burgdorferi*.

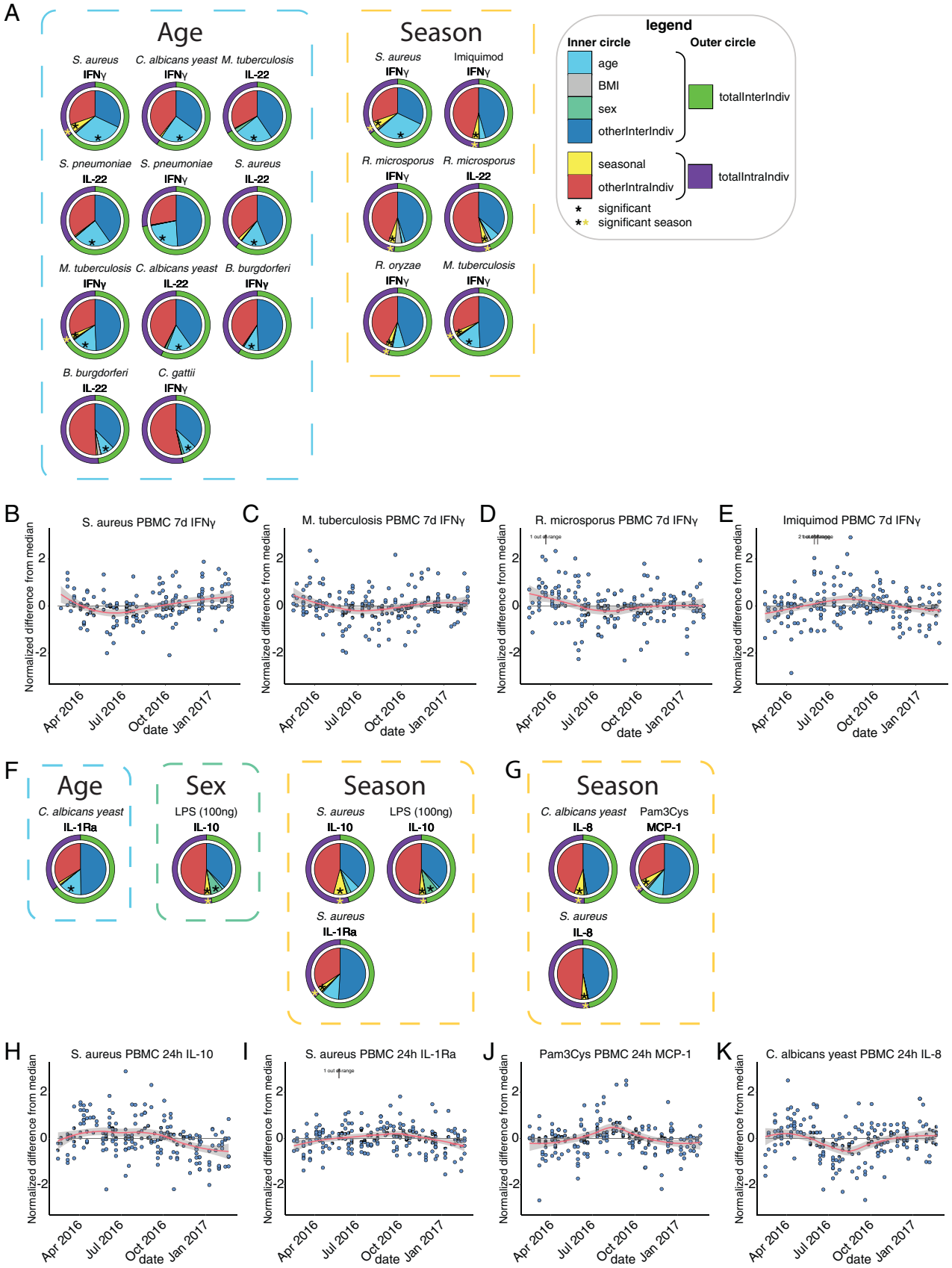

FIGURE 4. (**A**) Interindividual and intraindividual variation in long-term cytokine production capacity after stimulation of PBMCs. Only cytokine–stimulus pairs that showed significant association with age, sex, BMI, and season are shown. The pie charts are shown in a similar format as in Fig. 2A. (**B**–**E**) Example seasonality plots for several combinations of long-term cytokines and stimulations. In this plot, for each individual, the median value over all four timepoints was subtracted from all four timepoints. This way, we are removing (most of) the interindividual variation, leaving just *(Figure legend continues)*

although B cells did show some limited seasonality. There was little sex and BMI dependence for the cell subset counts.

In addition to the flow cytometry data, platelets, basophils, eosinophils, RBCs, hemoglobin, and hematocrit were measured using a differential hematology analyzer. Although platelet counts were relatively unstable, showing almost 50% intraindividual variation, the mean platelet volume was extremely stable over time, with an intraindividual variation of just 12% (Supplemental Fig. 4, Supplemental Table VIII). Additionally, mean platelet volume showed a significant seasonal pattern, peaking in July. As displayed in Supplemental Fig. 4, basophil count and basophil percentages also showed seasonal patterns.

CMV serology

A positive CMV serology has been associated with strong changes in T cell numbers, specifically with more $CD4^+$ and $CD8^+$ EM T cells and especially with $CD4^+$ EM T cells that re-express the naive-cell marker CD45RA (14). We measured CMV serology in the 56P cohort (data available for 52/56 individuals, 14 seropositive), and found that CMV serology explained roughly 10–20% of the total variance for $CD4^+$ and $CD8^+$ EM T cells, which is in line with Patin et al. (14) (Supplemental Table IX). Additionally, $CD4^+$ and $CD8^+$ effector cells were observed to have a similar amount of variance explained by CMV serology as the EM T cells. Whereas Patin et al. (14) found that $CD45RA^+$ EM T cells were much more dependent on CMV serology than $CD45RA^-$ EM T cells, we observed a similar profound effect of CMV serology on $CD45RA^-$ and $CD45RA^+$ EM cells. Finally, CMV serology had little to no effect on cytokine production capacity or circulating makers of inflammation (Supplemental Table IX).

Year-to-year variation

The inter- and intraindividual variation in the 56P cohort was calculated based on a 1-y period. Each individual in the 56P cohort was also part of the 500FG cohort, which was collected about 2 y prior (Fig. 1A). To determine the interindividual and intraindividual variation over a 2-y timeframe, we calculated the Pearson correlation between the values of 500FG cohort and the median values of the 56P cohort. One-year and two-year interindividual and intraindividual variation showed similar patterns for the circulating markers of inflammation (Fig. 6A), although the 1-y interindividual variation was higher than the 2-y interindividual variation for all markers. Conversely, 2-y intraindividual variation was higher than the 1-y intraindividual variation for all markers, as the interindividual and intraindividual variation together, by definition, sum to 100%. This suggests that as more time goes by, there is a larger drift in the concentration of circulating markers of inflammation within individuals. For cytokine production capacity, we observe a similar pattern, although short-term cytokine production showed a much weaker correlation between 1-y and 2-y interindividual variation compared with long-term cytokine production (Fig. 6B, 6C). Finally, cell counts showed a strong correlation between 1-y and 2-y interindividual and intraindividual variation, although overall interindividual variation is much greater over a 1-y period than a 2-y period (Fig. 6D). The pattern of a larger intraindividual variation and smaller interindividual variation as more time passes makes biological sense given that more time would create more opportunity for external factors to induce variation.

Genetic variation

Large percentages of the interindividual variation in cell subset counts and cytokine levels could not be explained by age, sex, CMV serology, or BMI. This unexplained interindividual variation is likely a combination of stable environmental factors (e.g., past and chronic exposure to pathogens) and genetic factors. Individual genetic makeup has been shown to be an important factor for many immune parameters (1, 3, 8–10, 14). Although our sample size is not sufficient to perform estimates of the genetic contribution to the interindividual variation, we previously calculated these percentages for a subset of the 56P cohort, measured at a single timepoint, called the 500FG cohort (Fig. 1B). These percentages are available for short-term and long-term cytokine production capacity (3) and cell counts (1). This allowed us to evaluate for which immune parameters genetic makeup explains the majority of the remaining interindividual variation. Fig. 6E shows the percentage of unexplained interindividual variance for the short-term cytokine production on the *x*-axis (i.e., interindividual variance not explained by age, sex, and BMI) and shows the percentage of variance explained by genetics on the *y*-axis. Fig. 6F shows the same type of plot for the long-term cytokines. If all unexplained variance is explained by genetics, that cytokine would be on the diagonal line, and anything below the line has remaining interindividual variance not explained by genetics. Genetic variation explained only a small part of the unexplained interindividual variance for both short-term and long-term cytokine production upon *C. albicans hyphae* and *C. albicans yeast* stimulation. The same holds true for the short-term cytokine production upon *Pam3Cys* stimulation. This means there are one or more unknown stable host factors that strongly influence cytokine production in response to these pathogens. In contrast, cytokine production in response to *C. gattii* had most of its remaining interindividual variation explained by genetics, meaning that together, age, sex, BMI, and genetics explained most of the differences between individuals. This was also true for short-term cytokine production in response to *S. aureus*. Fig. 6G shows that for cell subset counts, there was a large spread in the percentage of remaining variation explained by genetics.

Discussion

In the current study, we investigated immune responses in a cohort of 56 healthy volunteers sampled at four timepoints at 3-mo intervals. We quantified the interindividual and intraindividual variance for a wide range of immune parameters, including circulating markers of inflammation, cell counts, and the production capacity of different cytokines and chemokines. We were able to evaluate the relative contribution of age, BMI, sex, CMV serology status, and genetic variability to the interindividual variation and the contribution of seasonality to the intraindividual variation.

With respect to the circulating inflammatory markers, AAT showed the largest intraindividual variation, with a significant seasonal contribution to this variability. AAT concentrations peaked in winter (Fig. 2A, 2D), which is in line with our previous study (12).

the intraindividual variation. This strategy was applied just for the visualization of the seasonality, and the statistical analyses are described in more detail in *Materials and Methods*. The fitted line and error range were plotted using the LOESS method of the geom_smooth function of the ggplot2 package and, again, is purely for visualization. (**F**) Similar to (A), but now for anti-inflammatory short-term cytokines IL-10 and IL-1Ra. (**G**) Similar to (A), but now for the chemokines MCP-1 and IL-8. (**H–K**) Similar to (B), but now for short-term anti-inflammatory cytokines and chemokines.

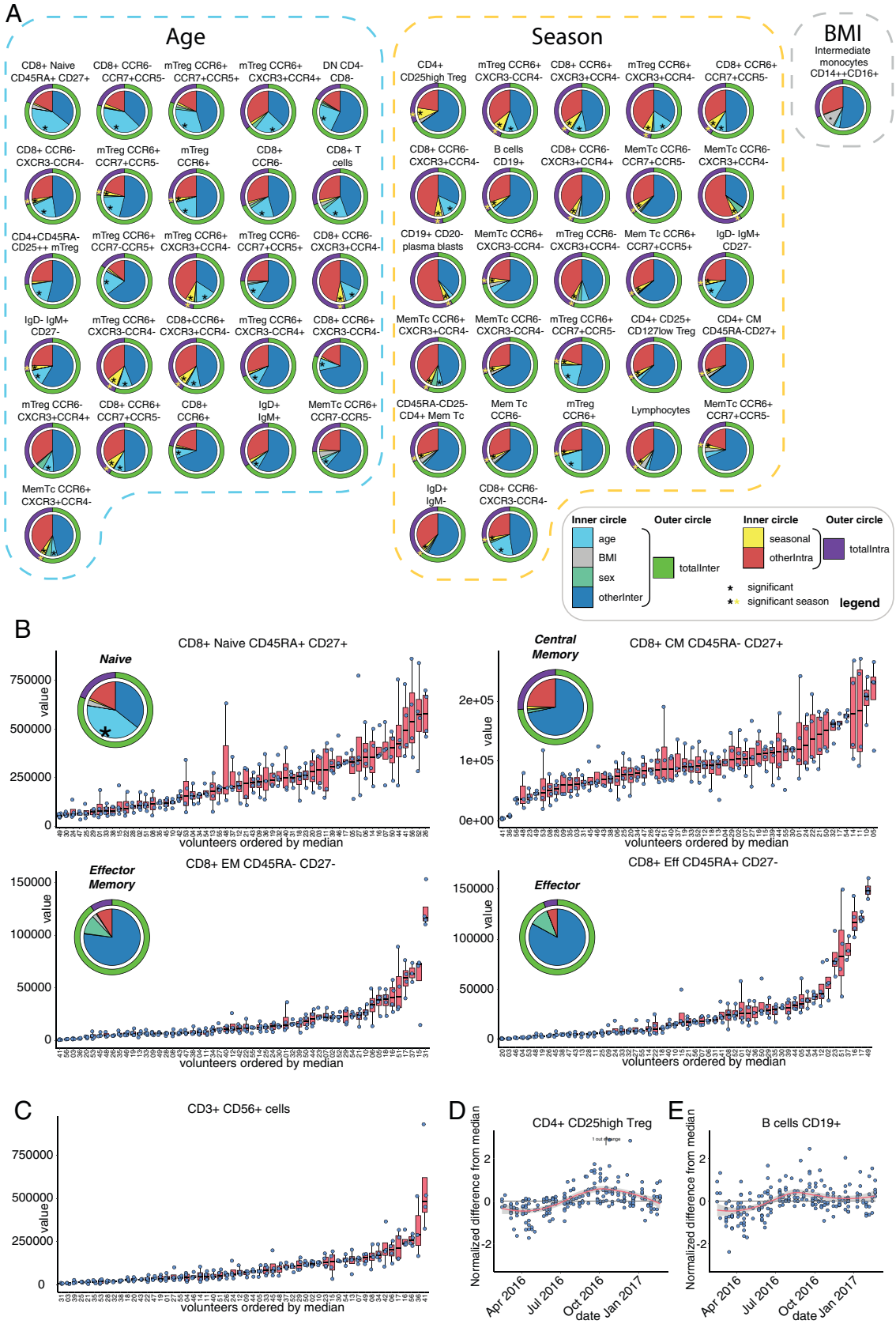

FIGURE 5. (**A**) Interindividual and intraindividual variation cell counts. Only cytokine–stimulus pairs that showed significant association with the age, sex, BMI, and season are shown. The pie charts are shown in a similar format as in Fig. 2A. (**B**) Change in explained variance as $CD8^+$ T cells mature from naive to effector cells, with example plots of its stability. Example plots show the individuals ordered by their median values, in which a boxplot is plotted with all individual measurements. Note that with just four measurements, a boxplot does not have a lot of meaning, so this is just for visualization purposes. (**C**) Stability of $CD3^+CD56^+$ T cells, similar plot as (B). (**D**) Example seasonality plot for $CD4^+CD25^{high}$ Tregs. In this plot, for *(Figure legend continues)*

Additionally, IL-18, IL-18BP, and resistin were moderately increased in winter. Interestingly, IL-18 has previously been shown to vary between the different seasons, peaking in autumn/winter, and this effect is thought to be independent of vitamin D levels (13). hsCRP had a large amount of nonseasonal intraindividual variation, suggesting that multiple measurements are needed to get a reliable estimate of someone's average hsCRP concentration in the circulation. This variation should be taken into account when using hsCRP as a biomarker for cardiovascular disease (19).

In addition to circulating cytokines, we measured the in vitro cytokine production after stimulation with different pathogens. The stability of short-term cytokine production capacity varied between stimuli. For example, short-term cytokine production in response to stimulation with *R. oryzae* was highly stable, whereas cytokine production in response to influenza was much more variable. This highlights the complexity of immune response regulation and how the likelihood of some pathogenic infections might be more dependent on intraindividual variation than others, either because of the season or other environmental influences. The seasonality of cytokine production in response to influenza is in line with the seasonal occurrence of influenza infections. In this study, we confirm with a longitudinal study that short-term cytokine production after stimulation with influenza is highly seasonal, as was previously shown in a cross-sectional study (12). However, the peak of cytokine production was shifted about 2 mo, from August (500FG) to October (56P) (Fig. 3B). Seasonal differences from year to year may explain this relative inconsistency. For instance, high summer temperatures started sooner and remained longer in the year in which the 56P cohort was collected compared with the 500FG cohort. Specifically, temperatures dropped significantly around August for the 500FG cohort, whereas they dropped in the middle of September to the beginning of October for the 56P cohort (Supplemental Fig. 5). As there are more seasonal parameters that differ from year to year (e.g., humidity, exposure to Ags, and pollen concentrations), causality cannot be proven. Cytokine production in response to *Borrelia* was also seasonal and dropped around July. This coincided with the annual increase of reported tick bites and the incidence of Lyme disease in the Netherlands (20). Given the limited number of people that are exposed each year, it is unlikely that seasonality in cytokine production solely results from exposure to the pathogen. Nevertheless, this variability may have consequences for the disease trajectory caused by a *Borrelia* infection.

Out of the three long-term cytokines evaluated in this study, IFN-γ and IL-22 have a larger percentage of interindividual variation than IL-17. This is mainly due to the strong influence of age on IFN-γ and IL-22, as previously reported (12). The similarities between IL-22 and IFN-γ likely result from genetic coregulation, given their proximity (of only ∼100 kB) on the genome, compared with IL-17, which is located on a different chromosome. We observed seasonal effects for IFN-γ production after several stimuli, with lower IFN-γ production in July. Interestingly, imiquimod stimulation showed an inverse pattern, with a peak in IFN-γ production around July (Fig. 4B–E). The cause of this difference is not known, although one may speculate whether this may be due to imiquimod being a pure TLR7/8 agonist, whereas most other stimuli that were used are whole pathogens that initiate a whole range of signaling cascades and receptor stimulations. IFN-γ production in response to Ag exposure has previously been linked to seasonal variation (21).

Interindividual variation in immune cell counts and, specifically, the influence of environmental and genetic factors have been topics of great interest. There have been conflicting results about whether stable host factors or environmental factors are the main source of variance in immune cell counts (4, 5, 7, 11). Regardless of which factor is dominant, age, sex, and season have all been shown to significantly influence immune cell counts (1, 4, 7, 9, 11). However, there has been a lack of longitudinal measurements, hampering our ability to accurately estimate inter- and intraindividual variation and the importance of seasonality. We observed that some of the larger cell subsets, like total leukocyte numbers, as well as lymphocytes, neutrophils, and monocytes, tend to have a relatively large amount of intraindividual variation compared with several of the smaller subsets of the main lymphocyte cell class, which are more stable over time. Especially $CD3^+CD56^+$ and NK cell counts are very stable over time. This is in line with previous findings that showed that out of all immune cells, NK cell numbers are most strongly influenced by individual genetic background (1).

As they mature, $CD8^+$ T cells showed changes in inter- and intraindividual variation. Naive $CD8^+$ T cells were strongly decreased with age; however, as they matured to central memory, EM and effector $CD8^+$ T cells, this age dependency was lost. Maturation to EM and effector $CD8^+$ T cells seems to be tightly regulated, as these cell numbers were extremely stable over time. Prior infection with CMV greatly influenced effector and EM cells, which explains part of their stability. One could speculate that other past infections and current chronic/latent infections might have further contributed to the stability of EM and effector cells. Within the $CD4^+$ T cells, we saw similar patterns, although both the age dependency of the naive cells and the stability changes in EM and effector were less pronounced. The stronger age dependence of $CD8^+$ naive T cells compared with $CD4^+$ naive T cells matches previous findings (14). Finally, we observed that $CD4^+CCR6^+$ memory Tregs increased with age, which confirms findings by Mo et al. (22) in mice.

It has previously been shown that particular immune cell subset counts vary between seasons (e.g., B cells) (1) (Fig. 5E). We found that $CD4^+CD25^{high}$ Tregs were most strongly influenced by seasonality, showing an increase around October (Fig. 5D). Surprisingly, we only observed significant seasonal effects for a limited number of immune cell subsets, and the explained variance is relatively low for most cell types when compared with cytokine production capacity. Monocyte counts, for instance, show no seasonal patterns. This suggests that the strong seasonal variation in short-term cytokine production is not driven by changes in cell counts, but rather by changes in their intrinsic function. The factors influencing the season-independent intraindividual variability of monocytes remain to be identified.

each individual, the median value over all four timepoints was subtracted from all four timepoints. This way, we are removing (most of) the interindividual variation, leaving just the intraindividual variation. This strategy was applied just for the visualization of the seasonality, and the statistical analyses are described in more detail in *Materials and Methods*. The fitted line and error range were plotted using the LOESS method of the geom_smooth function of the ggplot2 package and, again, is purely for visualization. (**E**) Similar to (D), but now for $CD19^+$ B cells.

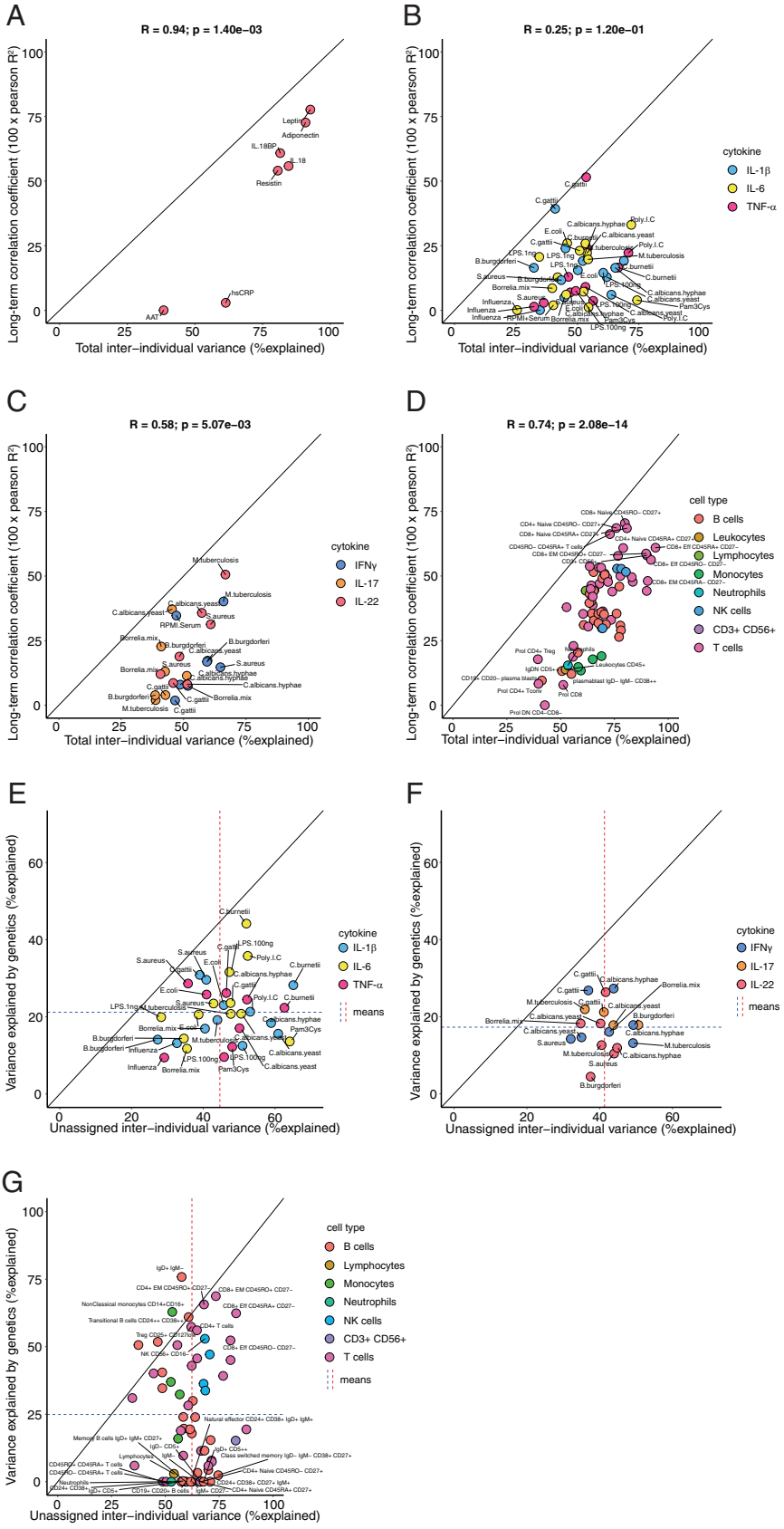

FIGURE 6. (**A**) Total interindividual variance for the circulating markers of inflammation as calculated within the 56P cohort on the *x*-axis versus correlation between the median value for each individual in the 56P and 500FG cohort on the *y*-axis. The former reflects stability over the course of 1 y, whereas the latter reflect stability over 2 y. (**B**) Similar to (A), but now for the short-term cytokine production capacity. (**C**) Similar to (A), but now for the long-term cytokine production capacity. (**D**) Similar to (A), but now for cell counts. (**E**) Interindividual individual variance not explained by age, sex, or BMI on the *x*-axis versus variance explained by genetics on the *y*-axis for short-term cytokine production capacity. (**F**) Similar to (E), but for the long-term cytokine production capacity. (**G**) Similar to (E), but now for cell counts.

In addition to environmental factors, genetic variation also shapes the immune variation between individuals. By separately estimating interindividual and intraindividual variation, we were able to calculate the fraction of the interindividual variability explained by genetics. In this study, the interindividual variance represents the maximum amount of variance that could potentially be explained by genetics. Fig. 6 shows that on average, genetics explained almost half of the interindividual variance of the cytokine production capacity that is not already explained by age, sex, and BMI. The genetic contribution varied highly between stimuli, from a negligible amount to nearly all of the unexplained variation. For instance, long-term cytokine production in response to *C. gattii* had <50% total interindividual variation, and almost all of this was explained by genetics. IL-6 production in response to LPS stimulation had ~30% interindividual variation, which for more than two thirds was explained by genetics. Overall, we observed a high contribution of genetics to the interindividual variation in cytokine production capacity. For immune cell counts, the unexplained interindividual variance ranged between ~35 and 90%. For some of these cells, (almost) all of this variance was explained by genetics (e.g., $CD4^+$ T cells and $CD4^+$ EM cells), whereas for others (almost) none of the variance was explained by genetics (e.g., lymphocytes and neutrophils).

The current study has several limitations. First, the duration of the study was 1 y, making the results specific to the environmental influences and conditions the volunteers encountered that specific year. However, we have no reasons to believe that the patterns during other years would be substantially different. Second, some degree of technical variability in our measurements is inevitable. A low signal-to-noise ratio for parameters with relatively low intensities (e.g., weak immune stimuli or low cell counts) could potentially artificially increase intraindividual variation. Therefore, we evaluated if interindividual and intraindividual variation were influenced by the intensity of the parameters and found no correlation (Supplemental Fig. 6). Third, if any of the volunteers suffered from chronic or intercurrent infections or used medication, this could influence the results. We therefore included several questions in the questionnaire regarding these topics and excluded volunteers for those timepoints for which they indicated recent or chronic disease and/or medication usage. After excluding these samples, we also checked for clear outliers in the circulating markers of inflammation and cell counts and found none. Ideally, a future study would also include technical replicates for each sample at each timepoint. Naturally, several of the immune data types that were assessed show associations (Supplemental Table X), and these associations are likely the result of the underlying biological mechanisms. However, the limited number of individuals in this cohort prevented a full analysis of these associations. A deeper characterization of the cellular source of the measured cytokines would be desirable in future studies. Finally, the genetic analyses considered only common genetic variants, leaving out any potential contribution of copy number variation, insertion, deletions, and rare variants, which should be a focus of future studies.

In conclusion, we identify sources of both interindividual and intraindividual variation in immune responses. Seasonality is an important environmental factor that influences several immune responses, and its effects are specific to certain pathogens and cell types. These data suggest that in addition to specific genetic and nongenetic host factors, seasonality influences immune responses and, as such, may well explain the variation in the incidence and severity of immune-mediated diseases during the year.

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Disclosures

The authors have no financial conflicts of interest.

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