Integration of multi-omics data and deep phenotyping enables prediction of cytokine responses

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The immune response to pathogens varies substantially among people. Whereas both genetic and nongenetic factors contribute to interperson variation, their relative contributions and potential predictive power have remained largely unknown. By systematically correlating host factors in 534 healthy volunteers, including baseline immunological parameters and molecular profiles (genomic, metabolome and gut microbiome), with cytokine production after stimulation with 20 pathogens, we identified distinct patterns of co-regulation. Among the 91 different cytokine–stimulus pairs, 11 categories of host factors together explained up to 67% of interindividual variation in cytokine production induced by stimulation. A computational model based on genetic data predicted the genetic component of stimulus-induced cytokine production (correlation 0.28–0.89), and nongenetic factors influenced cytokine production as well.

Variability in baseline immune response influences individuals’ susceptibility to immunologically mediated diseases such as infection, autoimmune and inflammatory diseases, as well as disease severity⁴–⁸. Because both environmental and host factors are responsible for this variation in immune response⁴–⁸, deciphering their interaction is crucial for understanding their influence on disease susceptibility and building quantitative predictors of disease. The Human Functional Genomics Project (HFGP) aims to identify the factors responsible for the variability in immune response in the general population and after perturbations, such as in disease states. Within the HFGP, the 500 Human Functional Genomics (500FG) consortium has collected extensive molecular and phenotypic measurements from approximately 500 healthy volunteers of Western-European descent. Earlier 500FG studies have assessed the separate effects of host-related factors, genetic variation and microbiome on cytokine production⁵–⁸. However, an integrated understanding of the effects of these factors and of additional host-related factors, such as endocrine hormones, circulating metabolites, platelet-mediated effects or transcriptional profiles of immune cells on stimulus-induced cytokine levels, has been lacking.

Here, we used a comprehensive systems biology approach to integrate the large-scale genomic, metagenomic and metabolomic data available within the 500FG consortium with the immune-cell composition, hormone levels and platelet-activation profiles of each person analyzed. This process allowed us to describe the baseline heterogeneity of immunological parameters, identify intercorrelated immunological components, infer functional connections within the immune system and build predictive models of cytokine production after stimulation. Using transcriptome data, we showed that expression of genes after stimulation explained the variation in cytokine production better than baseline expression. By integrating multi-omics layers, we showed that cytokine production is regulated by multiple genetic and nongenetic host factors, that production of cytokines after stimulation is moderately predictable through the use of multiple baseline profiles and that interindividual variation in immune responses correlates with individuals’ genetic risk of (auto)immune disease.

Results
Baseline immunological parameters are intercorrelated. To understand interindividual variation in the human immune response, we previously generated a database of immunological measurements, multi-omics data (cytokine-response profiles, genetics, gene expression, immune-cell frequencies, immunological modulators, immunoglobulins, hormone levels, blood platelets, circulating metabolites and gut microbiome composition) and classical phenotypes (age, sex and body mass index (BMI)) from volunteers in the 500FG cohort (Supplementary Fig. 1a,b and Supplementary Table 1). Cytokine production levels in individuals were assessed through previously generated enzyme-linked immunosorbent assay profiles including six cytokines (IL-1β, IL-17, IL-22, IL-6, TNF and IFN-γ) detected in peripheral blood mononuclear cells (PBMCs),
whole blood and PBMC-derived macrophages derived from blood after stimulation with 20 pathogens (Supplementary Table 2). IL-1β, IL-6 and TNF levels were measured 24 h after stimulation, and IL-22, IL-17 and IFN-γ levels (androstenedione, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone) were measured 7 d after stimulation. In whole blood, IL-1β, IL-6 and TNF levels were measured 48 h after stimulation.

To map the relationships among these different molecular and immunological parameters, we first performed clustering analysis of all immunological measurements beyond cytokine production. To decrease the dimensionality of the dataset, the first ten principal components (PCs), covering >75% of variance in each dataset, were individually extracted from the cell-count, metabolite and microbiome datasets. These PCs were then combined with measurements of immunological modulators (IL-18, IL-18BP , resistin, leptin, adiponectin and α-1 antitrypsin), immunoglobulins (IgG1–4, IgA and IgM), platelet-activation profiles (P-selectin expression, fibrinogen binding, coagulation markers and β-thromboglobulin) and hormone levels (androstenedione, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, progesterone, testosterone, 25-hydroxy vitamin D3, TSH and T4) (Supplementary Table 1). Subsequent unsupervised clustering analysis revealed several clusters (Fig. 1) that were consistent with previous observations, thereby validating the current correlations. We observed a negative correlation between the amount of the hormone leptin and the levels of progesterone and testosterone.
in peripheral blood (Fig. 1), a result consistent with an inhibitory effect of leptin on progesterone and testosterone in humans\textsuperscript{10–13}. We also observed a negative correlation of expression of p-selectin (whole-blood flow cytometry) and fibrinogen-activation profiles in peripheral blood (Fig. 1), results consistent with evidence that both are under shared control\textsuperscript{14,15}. Similarly, the hormone levels of 17-hydroxyprogesterone and testosterone were positively correlated with those of progesterone, androstenedione and 11-deoxycortisol levels in peripheral blood (Fig. 1), results consistent with these molecules having common synthesis pathways. Finally, we observed the clustering of α-1 antitrypsin with adiponectin and the association of two immune-cell-frequency PCs with total platelet count, as well as a negative association between IL-18 and IgM abundance (Fig. 1).

These results show that baseline immunological parameters in healthy individuals are correlated and are likely to be influenced by co-regulatory pathways.
Baseline molecular profiles show substantial variation. Next, we examined the baseline (unstimulated) interindividual variation in the immunological and molecular profiles described above and found a wide range of variation for most immunological parameters analyzed (Supplementary Fig. 1c–e). Because some variation is known to result from differences in age, sex and season\(^{9,16-19}\), we...
corrected for those effects, when applicable. Among the immune-cell populations with high variability, effector T cell subpopulations showed the largest interindividual variation, as compared with the other immune-cell subpopulations (Supplementary Fig. 1c), results

in agreement with previous observations. Baseline transcript abundance in whole blood also showed substantial interindividual variation (Supplementary Fig. 1d). The top 75 most variable transcripts were significantly enriched in 23 Gene Ontology terms related to innate immunity ($P<0.05$, determined with an online tool) (Supplementary Table 3), thus suggesting that the innate immune response was a major contributor to variations in transcript abundance. This analysis demonstrates that the baseline molecular profiles vary substantially among healthy individuals.

Genetics contributes the most to immune variation. To address the extent to which responses to a perturbation were affected by preexisting immune status, we first assessed the effect of host factors at baseline on cytokine production. Using a multivariate linear model (MVLM) to examine the percentage of variance explained by these factors, we found that genetic variation, as measured by single-nucleotide polymorphisms (SNPs), collectively explained most of the variation in stimulated cytokine production (average adjusted $R^2=0.18$) (Fig. 2a). In contrast, the gut microbiome, immune-cell counts, circulating metabolites and seasons displayed only moderate effects (average adjusted $R^2=0.061$, 0.057, 0.047 and 0.041, respectively) on most cytokine–stimulus pairs (Fig. 2a), and the concentrations of circulating immunoglobulins and inflammatory mediators or hormones, and platelet activation (whole-blood flow cytometry) generally had negligible effects (Fig. 2a,b). To evaluate the significance of the estimates of variation explained by genetics (VG), we performed 1,000 permutations of sample labels in the cytokine data and applied the analysis pipeline to the permuted data to obtain the empirical distribution of the estimates of VG (null distribution). We subsequently compared the estimate of VG from the 500FG data with the estimate of VG from the permuted data. In total, the estimates of VG in the 500FG were significant in 59 of 91 cases ($P<0.05$, Supplementary Table 4). For
Fig. 6 | Stimulated cytokine production correlates with genetic risk score for autoimmune diseases. a, Example individuals with high genetic risk of (auto)immune disease tend to be high producers of cytokines in response to pathogens. Asterisk indicates significant differences between low- and high-risk groups for T1D ($P = 0.011$ by two-tailed Wilcoxon rank-sum test). Low- and high-risk groups ($x$ axis) were selected by taking the top and bottom quartile of the PRS for T1D. The $y$ axis indicates the IL-6 level after stimulation of PBMCs with influenza. b, Distribution mean correlations between T1D risk in monocyte-derived cytokines (left) and lymphocyte cytokines (right) for 1,000 permutations. The measured estimate is indicated by the red arrow. T1D shows significance for monocyte derived cytokines (left) but not for the lymphocyte-derived cytokines (right). c, Distribution of Spearman correlation coefficients between stimulated cytokine production and genetic risk score for immune disease in 430 individuals, shown for PBMCs. Genetic risk scores were calculated on the basis of GWAS for different diseases. Significant differences in mean correlation between the lymphocyte- and monocyte-derived cytokines are shown ($^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$, NS, not significant by two-tailed Wilcoxon rank-sum test). Exact $P$ values: Crohn’s disease, $P = 7.28 	imes 10^{-6}$; eczema, $P = 2.55 	imes 10^{-5}$; inflammatory bowel disease, $P = 9.34 	imes 10^{-4}$; multiple sclerosis, $P = 4.85 	imes 10^{-4}$; psoriasis, $P = 1.40 	imes 10^{-4}$; rheumatoid arthritis, $P = 1.41 	imes 10^{-2}$; type 1 diabetes, $P = 1.00 	imes 10^{-3}$; type 2 diabetes, $P = 1.65 	imes 10^{-3}$; ulcerative colitis, $P = 1.34 	imes 10^{-3}$. Center lines show the medians; box limits indicate the twenty-fifth and seventy-fifth percentiles, as determined in R software; whiskers extend to 1.5 times the interquartile range from the twenty-fifth and seventy-fifth percentiles; outliers are represented by dots outside the whiskers.

**example**, we found that the cytokine–stimulus pairs that were best explained by genetics (polynosinic–polycytidylic acid (polyI:C)-induced and *Coxiella burnetti*-induced IL-6 levels in PBMCs) showed significance.

**Furthermore**, we assessed several specific baseline categories that showed cytokine or pathogen specificity in explaining the interindividual variation (Fig. 2b). We observed that the abundance of circulating metabolites, including acetate and high-density lipoprotein (HDL) cholesterol, showed a moderate negative effect on influenza-stimulated cytokine production by PBMCs (average adjusted $R^2 = 0.19$) (Fig. 2b), thus suggesting that these factors modulate susceptibility to viral infections. The production of the lymphocyte-derived cytokines IL-17, IL-22 and IFN-γ by PBMCs in response to *Aspergillus fumigatus conidia* was driven more by nongenetic host factors (cell counts, platelet amounts, circulating metabolites, gut microbiome composition and season) than by genetic factors (Fig. 2b), a result in contrast to the gene-component-driven cytokine production in response to all other stimulations used (Fig. 2b). More specifically, individuals with high concentrations of HDL cholesterol or α-1 antitrypsin in the circulation showed lower cytokine production in response to *A. fumigatus*. To validate the link between HDL cholesterol and cytokine production, we cultured PBMCs collected from six healthy volunteers in medium containing lipoprotein-deficient plasma with or without HDL cholesterol, and measured production of the cytokines TNF, IL-1β and IL-6 in response to *A. fumigatus conidia* after 24 h. We observed lower production of all the cytokines assessed in PBMCs cultured with HDL compared with the lipoprotein-deficient plasma control (Supplementary Fig. 2a), thus indicating that HDL cholesterol modulates immune responses to *A. fumigatus conidia*.

**Next**, we compared the data on stimulus-dependent cytokine production from the three different types of stimulation assays (PBMCs, whole blood and PBMC-derived macrophages) from the same individuals. We found that season, platelet-activation profiles, concentration of immunological modulators and age had greater effects on stimulus-dependent cytokine production in PBMCs than in macrophages (Fig. 2a,b). In contrast, stimulus-dependent cytokine production was less correlated with baseline metabolite levels in PBMCs and whole blood than in macrophages (Fig. 2a,b).
This analysis shows that genetics substantially contributes to the observed interindividual variation in cytokine levels after stimulation, and the nongenetic molecular profiles and immune parameters also contribute.

**Baseline molecules are differentially associated with cytokine response.** We next assessed which baseline immunological and molecular components contributed most to variation in stimulus-induced cytokine production. We extracted the top five immunological parameters and molecular profiles (immune-cell frequencies, immunological modulators, immunoglobulins, hormone levels, blood platelets, circulating metabolites and gut microbiome composition) to add to the model (n = 353 individuals). In this analysis, circulating baseline (unstimulated) measurements of IL-18BP were determined. Because this cohort comprises mainly obese (BMI >25) and older (age >55) individuals, we limited the analysis to a subset of (n = 51) 300-OB volunteers with BMI <28, to make this distribution more in line with that of the 500FG cohort. We tested for association (Spearman correlation) between the cytokine production profiles after stimulation and the circulating IL-18BP levels (Supplementary Fig. 2b). We were able to replicate the negative effect of IL-18BP on lymphocyte cytokines.

The short-chain fatty acid (SCFA) acetate showed the strongest correlation (negative correlation between −0.25 and −0.20) with influenza-induced monocyte-derived IL-1β, IL-6 and TNF cytokine production (Fig. 3). Cytokine response to bacterial and fungal stimulation showed either positive or negative effects on the production of monocyte-derived cytokines. In contrast, lymphocyte-derived IL-17, IL-22 and IFN-γ cytokine production showed consistently positive effects in response to most of the bacterial and fungal stimulation conditions. These results are consistent with previous findings that SCFAs, including acetate, influence cytokine production14-26. The negative correlation between acetate and stimulus-induced production of IL-1β, IL-6 and TNF was also observed in PBMC-derived macrophages but not in whole blood (Fig. 3). To further investigate the association between acetate and stimulus-induced cytokine production, we cultured PBMC-derived macrophages obtained from the whole blood of six healthy Dutch volunteers in vitro in the presence of acetate in the medium, stimulated them with Mycobacterium tuberculosis, Candida albicans, Staphylococcus aureus or Escherichia coli, and assessed the production of the cytokines TNF and IL-6 after 24 h. We observed an association between acetate and cytokine production in macrophages after stimulation (negative correlation between −0.25 and −0.20) with influenza-induced monocyte-derived IL-1β, IL-6 and TNF cytokine production (Fig. 3). Cytokine response to bacterial and fungal stimulation showed either positive or negative effects on the production of monocyte-derived cytokines. In contrast, lymphocyte-derived IL-17, IL-22 and IFN-γ cytokine production showed consistently positive effects in response to most of the bacterial and fungal stimulation conditions. These results are consistent with previous findings that SCFAs, including acetate, influence cytokine production14-26. The negative correlation between acetate and stimulus-induced production of IL-1β, IL-6 and TNF was also observed in PBMC-derived macrophages but not in whole blood (Fig. 3). To further investigate the association between acetate and stimulus-induced cytokine production, we cultured PBMC-derived macrophages obtained from the whole blood of six healthy Dutch volunteers in vitro in the presence of acetate in the medium, stimulated them with Mycobacterium tuberculosis, Candida albicans, Staphylococcus aureus or Escherichia coli, and assessed the production of the cytokines TNF and IL-6 after 24 h. We observed an association between acetate and cytokine production in macrophages.
in which the production of TNF and IL-6 in PBMC-derived macrophages after exposure to two of the stimuli (E. coli and S. aureus) was lower in the presence of acetate, but this effect was not observed for C. albicans (Supplementary Fig. 2c).

Glutamine is known to negatively regulate IL-6 production in human intestinal mucosa27 and to decrease IL-6, TNF and IL-1β production in biopsies from people with Crohn’s disease16. We observed that glutamine was consistently negatively correlated with all monococyte- and lymphocyte-derived cytokines assessed after stimulation (Supplementary Fig. 3) and consequently might be used as an anti-inflammatory biomarker. These results show that baseline molecules are differentially associated with cytokine production between stimuli, as well as between cell types.

Host factors explain up to 67% variation in cytokine levels. To determine the collective contribution of genetic variation and immunological components at baseline to cytokine production in response to pathogens, an MVLM was used. We constructed an MVLM for each cytokine–stimulus pair, in which we added relevant features from each category of dataset sequentially, then evaluated the increase in variance explained by each added dataset. This integrated approach indicated that a combination of genetics, baseline molecular profiles and immunological parameters explained up to 67% of the interindividual variation in cytokine production (Fig. 4). Because cytokine production is a highly complex phenotype, and many factors that influence it are themselves associated, we tested whether changing the order in which specific datasets were added into the models might generate different results. When we compared the MVLM containing all datasets and the partial MVLMs, in which each of the ten datasets was omitted once, we found estimates of explained variation similar to those in the sequential analysis (Supplementary Fig. 4). For example, regardless of the order in which the factors were added, genetics remained the largest individual contributor to explaining interindividual variation (Supplementary Fig. 4). This result indicated that the order in which various factors were added into the model did not influence the results to a large extent.

Gene expression correlates with cytokine response. Next we integrated baseline transcript abundance with stimulus-induced cytokine expression. We made use of whole-genome gene expression profiles obtained with RNA-seq both before and after stimulation of peripheral blood with C. albicans conidia from a subset of volunteers (n = 64) from an independent Dutch cohort (Genome of the Netherlands cohort30). We used measurements of the production of TNF, IL-6 and IL-1β by PBMCs after stimulation with C. albicans conidia after 24 h in the same individuals. We then applied the same MVLM-based analysis approach used earlier to obtain estimates of how much interindividual variation in cytokine production capacity could be explained by gene expression. We observed that baseline gene expression explained a substantial portion of the interindividual variation in production of TNF, IL-6 and IL-1β (Fig. 5). Production of TNF; IL-6 and IL-1β by PBMCs stimulated with C. albicans conidia showed significantly higher correlations with gene expression induced by stimulation (adjusted R² up to 0.75) than with baseline gene expression (two-tailed Wilcoxon rank-sum test, P = 1.08 × 10⁻³, P = 8.93 × 10⁻³, for TNF, IL-6 and IL-1β, respectively). Using Gene Ontology enrichment (online tool20), we found that the genes selected during modeling (Supplementary Table 5) showed enrichment for several Gene Ontology terms related to immune responses. For example the genes associated with C. albicans–induced TNF levels were nominally enriched in negative regulation of mast cell cytokine production (P = 1.28 × 10⁻³), negative regulation of isotype switching to IgE isotypes (P = 1.71 × 10⁻³) and negative regulation of type 2 helper T cell differentiation (P = 2.15 × 10⁻³). These results suggest a strong correlation between gene expression and functional responses after stimulation with pathogens and thus suggest gene expression as a target for future studies on the prediction of immune responses.

Immunological disease risk is associated with stimulated cytokine levels. Because many complex diseases appear to result from multiple genetic variants exerting small effects on disease risk31,32, complex diseases may conform closely to a classical polygenic model. Using publicly available summary statistics from genome-wide association studies (GWAS), we calculated polygenic risk scores (PRSs) for 15 immunologically mediated diseases (Supplementary Table 6) for all the volunteers in the 500FG cohort, as a measure of relative disease risk between individuals. We then tested whether volunteers with a higher risk of an immunologically mediated disease displayed higher or lower stimulus-induced cytokine production than the lower-risk individuals. For this analysis, we focused on the immunologically mediated diseases that showed both a significant change (two-tailed two-sample t test, Bonferroni P < 0.05; Supplementary Table 7), compared with a permutation-based null distribution, and a consistent pattern at different thresholds used for PRS calculation.

Fig. 8 | Prediction using the genetic model in an independent dataset shows that some cytokine–stimulus pairs can be predicted successfully. Spearman correlations between predicted cytokine level by the MVLMs built by using genetics (n = 336) and the measured values in an independent set of stimulation experiments (n = 56). The box plots show the variations in Spearman correlations from each of the ten MVLM predictions from the cross-validation strategy. Center lines show the medians; box limits indicate the twenty-fifth and seventy-fifth percentiles, as determined in R software; whiskers extend to 1.5 times the interquartile range from the twenty-fifth and seventy-fifth percentiles; outliers are represented by dots outside the whiskers.
Stimulated cytokine levels predicted by genetics. Finally, we integrated both genetics and other molecular features to construct MVLMs to predict each cytokine–stimulus pair in PBMCs, whole blood and macrophages. To achieve the best prediction of ex vivo stimulus-induced cytokine production, we tested several linear prediction methods (Elastic Net, rrBLUP and PLS) and compared them by using both genetic and nongenetic factors to train the MVLMs for each cytokine–stimulus pair. Predictive performance was quantified by Spearman’s correlation between the measured and the predicted stimulus-induced cytokine production in multiple randomly selected subsets of the volunteers from 500FG. Whereas the prediction performances of the different methods were similar (Supplementary Fig. 6a–c), Elastic Net marginally outperformed the others, so we used it for subsequent analyses.

We first tested whether SNP data could predict cytokine production. Among the 91 cytokine–stimulus pairs, the correlations between predicted and measured stimulus-induced cytokine production were 0.69 on average (range 0.28–0.89) (Fig. 7a). Inclusion of the baseline immunological parameters and multi-omics data significantly increased the predictive power and stability of the model (two-tailed Student’s t test, P = 1.36 × 10^{-5}, t statistic = 6.09, degrees of freedom = 1,792), and most predictions for cytokine production increased to 0.72 on average (range 0.35–0.90) (Fig. 7b). Additional inclusion of the gene expression data from the RNA-seq analysis decreased the predictive power (average 0.60, range 0–1) (Supplementary Fig. 6d), probably as a result of the lower number of samples for which both RNA-seq and the other factors were available (n = 69).

We then tested the predictive capabilities of the Elastic net–trained MVLMs, using only SNPs as input, which were applied to an independent subset of 500FG individuals for whom new cytokine-stimulation experiments were performed (50FG). We found prediction accuracies as high as 0.56 for some cytokine–stimulus pairs (Fig. 8), although the MVLMs performed poorly for most stimulations. Among the best-performing cytokine–stimulus pairs, C. burnetti-stimulated IL-1β and polyI:C-stimulated IL-6 yielded prediction accuracies of 0.56 and 0.46, respectively, on average (Fig. 8). Because both pathways are known to have a large genetic component, we concluded that the MVLMs were able to predict cytokine production for stimulus-induced cytokines whose mechanisms of induction are primarily driven by genetics.

By applying MVLMs to genetic data, we were able to predict the cytokine production after stimulation, with varying degrees of accuracy.

Discussion
In this study, we assessed the combined contribution of genetic and nongenetic factors to the interindividual variations in cytokine production in response to pathogens, by examining the cytokine production of immune cells after stimulation with 20 different pathogens or Toll-like-receptor ligands ex vivo in PBMCs, whole blood and PBMC-derived macrophages. This analysis identified new modulators of cytokine production, including circulating inflammatory mediators and metabolites. We found that volunteers with elevated genetic risk of immunologically mediated diseases were more likely to be high responders in terms of stimulus-induced cytokine production. Finally, we trained MVLMs that successfully predicted human stimulus-induced cytokine production for polyI:C-induced IL-6 and C. burnetti-induced IL-1β levels in PBMCs by using only the genetic profiles or a combination of genetic and other molecular profiles.

A recent study on the heritability of immunological phenotypes in 210 twins has suggested that variations in circulating cytokine concentrations are primarily driven by nonheritable influences10. Although we observed here that genetics was the largest single contributor to interindividial variation (average adjusted R^2 = 0.18), most of the variation could still be explained by nongenetic influences. Any differences that we observed in estimates of heritability are likely to be due to differences in the experimental design between the two studies. We assessed cytokine profiles after stimulation ex vivo, whereas the above study measured baseline circulating concentrations in vivo. These results strongly suggest that the response to pathogens during infection is under stronger genetic pressure than the background level of mediators in the circulation. Our findings thus are in agreement with the idea that infections have a strong selective effect on the genetic control of immune responses10–12.

The present study has potentially important implications for understanding of the human immune response. We found that acetate, a circulating metabolite, was associated with changes in stimulus-induced cytokine production and especially in the modulation of type 1 and type 17 helper T cell responses. SCFAs such as acetate, propionate and succinate are released by the gut microbiome, and current literature suggests that SCFAs have important immunomodulatory properties24–27. We show here that acetate has similar effects in humans in vivo. Therefore, investigating the broader effects of SCFAs and identifying which microbiome profiles modify SFCA concentrations in the circulation should be important. We found a strong inhibitory effect of acetate on influenza-stimulated cytokine production, a phenomenon that deserves further scrutiny. Another important metabolic pathway that strongly influenced cytokine responses was the cholesterol- and lipoprotein-synthesis pathway. Cholesterol pathways have been described to have important immunomodulatory effects, and the level of cholesterol sulfate, a derivative of membrane cholesterol, has been shown to influence immunological processes such as T cell–receptor signaling and thymic selection11. Here, we showed that HDL cholesterol negatively affects influenza and Aspergillus-stimulated cytokine production, and possibly has important effects on the pathophysiology of these infections.

The ability to calculate prediction scores for specific immunologically mediated diseases and to link them to cytokine production shows that certain stimulus-induced cytokine profiles may contribute to particular diseases, for example, the capacity to release high amounts of monocyte-derived cytokines in T1D. Although we acknowledge that our power to detect these smaller associations is relatively limited, our approach can be used to link any given phenotype to disease scores when individual-level data are available. This method offers an opportunity to identify immunological pathways important in disease, which may serve as new therapeutic targets.

A second limitation of the 500FG cohort is that it contains a higher proportion of young people than the general population10 and therefore could have introduced age bias into the MVLM predictions. Although we acknowledge that the performance of
the MVLM prediction may vary in populations with different age ranges, BMI ranges or ancestry, our study provides a proof of concept that stimulus-induced cytokine production can be moderately predicted. Future studies in larger general population cohorts with greater ranges of age and ancestry should contribute to the generation of models with improved predictive potential for a general population. Future studies should also aim to extend the current analysis, which was limited to common SNPs (minor allele frequency >0.1), to include rare variants and mutations, a broadening of scope likely to further increase the observed effects of genetics on cytokine production after stimulation.

In conclusion, we present the most comprehensive assessment to date of the host factors that influence cytokine production. We show that genetics is a major contributor to the interindividual variations in cytokine production after pathogen stimulation. However, other nongenetic factors also influence cytokine production in response to most stimuli, including gut microbiome composition, numbers of immune cells in circulation and circulating metabolite concentrations. Individuals with elevated genetic risk of a given immune disease tended to have elevated cytokine production, and stimulus-induced cytokine production was able to be predicted for polyI:C-induced IL-6 and C. burnettii-induced IL-1β levels. This study provides the fundamentals for predicting components of cytokine production on the basis of genetics and baseline host-factor profiles, thus paving the way toward personalized immunologically based therapies.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41590-018-0121-3.

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Author contributions
Y.L., C.W. and M.G.N. designed the study. M.O., S.P.S., M.I., R.T.N.-M., H.J.P.M.K., I.J., R.J.X. and L.A.B.J performed the experiments and processed the data. U.V. collected and preprocessed public summary statistics. O.B.B. performed statistical analysis with assistance from R.A.-G., S.S., U.V. and L.F.; O.B.B., M.Z., Y.L., S.W., V.K., M.G.N., and C.W. interpreted the data. Y.L., C.W., M.G.N. and O.B.B. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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Methods

Study cohort. The main analyses were performed in the 500FG cohort, which is part of the Human Functional Genomics Project. This cohort consists of 534 healthy individuals (237 males and 296 females) of Caucasian origin. Volunteers ranged from 18 to 75 years of age, and most (421 individuals) were 50 years or younger (Supplementary Fig. 1a). The volunteers had BMI within normal limits (15 to 35), and most (380 individuals) had a BMI between 20 and 25 (Supplementary Fig. 1b). Of these 534 original volunteers, 45 were excluded because of genetic background and questionnaire results (medication usage and chronic disease), thus leaving 489 individuals.

Replication cohort. Validation experiments were performed in the 300-OB cohort. This cohort consists of ~300 Dutch individuals. All individuals had a BMI >25. The average BMI was 31, and the age ranged from 55 to 80 years, with an average age of 67 years. Validations were performed in a subset of the 300-OB cohort with an BMI ≤28 (n = 55). Circulating metabolites and mediators as well as stimulated cytokine levels were measured in the same way as in 500FG.

Experimental procedures. The experimental procedures used to measure levels of cytokines, modulators, immunoglobulins and hormones have been described previously. Genotyping, metagenomic sequencing of the gut microbiome, flow-cytometric sorting of PBMCs and determination of platelet-activation profiles have also been described previously. We selected a representative subset of 89 samples from the 500FG cohort for RNA-seq (balanced for age and sex to match the original distribution in the cohort). These samples were processed for sequencing with an Illumina TruSeq version 2 library preparation kit. Paired-end sequencing of 2 × 50-bp reads was performed with the Illumina HiSeq 2000 platform. The quality of raw reads was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Read alignment was performed in STAR 2.3.0 (ref. 43), and aligned reads were sorted with SAMTools. Gene-bioinformatics.babraham.ac.uk/projects/fastqc/). Read alignment was performed in STAR 2.3.0 (ref. 43), and aligned reads were sorted with SAMTools. Gene-

Statistical methods. Data prefiltering. After preprocessing, the gene expression, SNP, metabolite and microbiome datasets were filtered to remove any nonsignificantly associated features. This process was done to increase the efficiency of downstream analysis. The gene expression metabolite and microbiome datasets were correlated to all of the cytokine measurement, and all features showing a Spearman correlation with a Benjamini–Hochberg adjusted P < 0.05 to at least one cytokine were kept. This procedure yielded a dataset of 4,499 genes, 205 metabolites, 509 microbial pathways and 162 microbial taxonomies. The genetic variants were filtered with previously generated cytokine QTL profiles by setting the P-value cutoff at various thresholds depending on the application. To calculate the variance explained by genetics, a P-value threshold of P < 5 × 10^-8 was chosen. For prediction with the Elastic Net model, various thresholds were evaluated, after which all SNPs with P < 5 × 10^-8 were included in the analysis.

Estimation of explained variance. The estimation of variance explained by each of the data levels on the different stimulated cytokine production profiles was performed through application of a correlation-based feature-selection approach. In this approach, we built a model for each stimulated cytokine measurement in which only features associated with this measurement were included in the model. We selected these features by first regressing out the effects of age and sex, then associating the features in a data level to the current cytokine–stimulus pair.

If a feature showed a significant association (Spearman P value <0.05), the feature was included in the set of potential predictors. After all the associations had been computed, the set of potential predictors was correlated to itself to identify collinearity among this predictor set. If features within this predictor set showed an association (Spearman correlation >0.4), the feature that showed the least association (on the basis of correlation P values) to the cytokine–stimulus pair was removed. This procedure yielded a unique set of predictors for every cytokine–stimulus pair, which was then used to fit a multivariate linear model to estimate the variance explained by these features for that cytokine–stimulus pair. To account for the inflation that adding predictors had on the explained variation, the adjusted R^2 was taken as the measure of explained variance.

Permutation of cytokine GWAS. The baseline cytokine GWAS was performed as described previously. We randomly permuted the cytokine and covariate datasets 1,000 times, then ran the GWAS with these datasets to obtain 1,000 random profiles for each cytokine–stimulus pair. For each run, we obtained the QTL profile and estimated the explained variance with the permuted cytokine and covariate dataset and the pipeline described above. This procedure yielded a distribution of 1,000 estimates of explained variance for each cytokine–stimulus pair. A measured estimate was considered significant if it was in the top 5% of the permuted distribution of estimates for that cytokine–stimulus pair.

Estimation of age and sex effects. Age and sex effects on cytokine production were assessed by fitting univariate linear models for each cytokine–stimulus pair with age and sex as the independent variables, respectively. The R^2 was taken as the measure of explained variation of these models.

Estimation of seasonal effect. The effect of season on stimulated cytokine production was assessed with a linear combination of sine and cosine terms with the same period (equation (1), as described by ter Horst et al.):

\[ y = \beta + \alpha \sin \left( \frac{2\pi x}{365} \right) + \alpha \cos \left( \frac{2\pi x}{365} \right) + \epsilon \]  

(1)

where \( y \) represents the response (cytokine level), \( \beta \) represents the estimated intercept, \( \alpha \) represents the estimated predictor effect, \( x \) represents the day of the year on which the sample was taken, and \( \epsilon \) represents the residual effect.

Estimation of cumulative explained variance. To assess the proportion of variance that could be explained by all levels cumulatively, individual levels were added to a multivariate linear model one by one, and the total model adjusted R^2 was calculated for each step. If adding a level showed an increase in the total adjusted R^2 of the model, this value was extracted. To assess the contribution of each level conditional upon the others, the full model was fit first. Subsequently, several reduced models were fit in which one data level was missing. The adjusted R^2 of this full model was then compared against the model with the missing level. The difference between the reduced model and the full model was taken as a measure of the variance explained by that level when accounting for the effects of the other levels.

Cytokine-level prediction. Our objectives were to investigate whether genetic variants might reveal predictive insights into the cytokine production after stimulation and whether baseline immunological parameters, which are treated as quantitative phenotypes that are continuously distributed over a population, could improve predictive power for cytokine production after stimulation. In our population-based study, we searched for those subsets of genetic variants and immunological components that were most predictive of the various stimulated cytokine production profiles, rather than with exclusively those variants meeting a stringent level of statistical significance.

We assessed the validity of this approach by applying multiple methods, each of which is discussed in detail below. In total, three datasets were evaluated: one for predicting stimulated cytokine production with only SNPs, one containing all levels except gene expression and one with all levels including gene expression. First, features with little association with cytokine production levels (Spearman P >0.05) were removed for building the prediction models. For the SNP dataset, all SNPs with an association with a cytokine–stimulus pair with P < 5 × 10^-8 were used as input for feature selection. No filtering for collinearity was applied, because Elastic Net accounts for potential collinearity among predictors.

Elastic Net. Prediction of the cytokine levels was facilitated by training an Elastic Net model. A 2 × tenfold cross-validation approach was used, in which the data were first split into ten random training and test sets to validate the prediction, and the training set was then split once more for feature selection. Prediction accuracy was evaluated by calculation of Spearman correlations between the measured cytokine levels and the predictions of the Elastic Net model on the test sets.

rBLUP. To show that the prediction results were not influenced by a large extent by the methodology, a mixed linear model (equation (2)), as implemented in the package rBLUP, was applied:

\[ y = \mu + Z u + e \]  

(2)

where \( y \) represents the response (cytokine level), \( \mu \) is a vector of ones, \( \mu \) is the overall mean of the training set, \( Z \) is the matrix of predictors (traits), \( u \) is the random effect of the predictors, and \( e \) is a vector of residual effects. Predictions were made with tenfold cross-validation. Spearman correlation was then calculated between predicted and measured values. We applied this model as described previously.

Partial least-squares regression. In addition to the Elastic Net and rBLUP, a partial least-squares model was applied. Models were validated with tenfold cross-validation. Prediction of cytokine levels on the test set was done with a linear model (equation (3)):

\[ y = \beta + a x + e \]  

(3)

where \( y \) represents the response (cytokine level), \( \beta \) represents the intercept, \( a \) represents a vector containing the coefficients from the model, \( X \) represents the matrix of predictors (immune traits), and \( e \) represents the residual error.

Polygenic risk scores. We carried out polygenic scoring of disease risk with publically available GWAS results. Quantitative scores were computed for each trait in this study on the basis of the set of SNPs with P values lower than predefined P-value thresholds (pTs) in the GWAS. Multiple pTs were evaluated (pT < 5 × 10^-8,
1 × 10^{-3}, 1 × 10^{-4}, 1 × 10^{-5} and 1 × 10^{-10}). Throughout this work, we refer to the scores defined at \(p_T < 1 \times 10^{-3}\) as PRS. Full association summary statistics were downloaded from several publicly available resources\(^{44-52}\), as indicated in Supplementary Table 6. Studies done exclusively in non-European cohorts were omitted. Filters applied to the separate data sources are indicated below. All the dbSNP rs numbers were standardized to match those of GIANT 1 kG p1v3, and the directions of the effects were standardized to correspond to the GIANT 1 kG p1v3 minor allele. SNPs with different opposite-strand alleles compared to GIANT alleles were flipped. SNPs with A/C and C/G SNPs and SNPs with different alleles GIANT 1 kG p1v3 (tri-allelic SNPs, indels and unknown alleles) were removed from the analysis. Genomic control was applied to all \(P\) values for the datasets not genotyped by Immunochip or Metabochip. We calculated PRS by first clumping variants according to the threshold \(p_T\), linkage disequilibrium (\(R^2 < 0.2\)) and a 250-kb window with the PLINK 1.9 option ‘clump’, and exclusively European samples from 1000 Genomes data were used as a reference for linkage-disequilibrium calculation. PRS were subsequently obtained for each threshold \(p_T\) by calculating them with the linkage-disequilibrium-clumped subset of SNPs with the PLINK 1.9 option ‘score’.

Association between polygenic risk scores and cytokine production. The association between the PRS and cytokine production after stimulation was determined by calculating the Spearman correlation between each of the PRS profiles and each of the stimulated cytokine profiles. To evaluate the statistical significance of association, a permutation method was used. The cytokine data were permuted 1,000 times, and the correlation was calculated for each of these permuted datasets. Both the measured and permuted distributions were separated into the lymphocyte and monocyte groups, and Student’s \(t\) test was applied between the measured distribution and the permuted distribution. When either the monocyte or lymphocyte group showed a significant deviation from the permuted the measured distribution and the permuted distribution. When either the monocyte or lymphocyte group showed a significant deviation from the permuted distribution, a permutation method was used. The cytokine data were permuted calculating the Spearman correlation between each of the PRS profiles and each

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**Statistical parameters**

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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**Software and code**

Policy information about availability of computer code

**Data collection**

In total 534 adult volunteers from Nijmegen, the Netherlands (237 males and 296 females, age range 18–75 years) where included in 500FG. After visiting the hospital to donate blood, the volunteers received an extensive online questionnaire about lifestyle, diet, and disease history. Based on the results of this questionnaire 45 volunteers where excluded for various reasons, e.g., they were under medication, non-European ancestry, or had a chronic disease. By excluding these individuals from the analysis we minimized false positive effects on the cytokine production capacity in vitro and in vivo.

**Data analysis**

The analyses where performed using the statistical programming language R and several publicly available and previously published methods/library's. No custom algorithms have been developed in this study and all analyses were done using either base R 3.2 or previously published methods. Further software that was used consisted of the open source toolkits PLINK 1.9, FastQC 0.11, STAR 2.4 and SAMTools 1.4

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The data that support the findings of this study are available at https://hfgp.bbmri.nl/ were it has been meticulously catalogued and archived at BBMRI-NL aiming for maximum reuse following the FAIR principles, i.e., Findability, Accessibility, Interoperability, and Reusability. Individual level genetic data as well as other privacy sensitive datasets are available upon request at http://www.humanfunctionalgenomics.org/site/?page_id=16. These datasets are not publicly available because they contain information that could compromise the research participants privacy. The central data stewardship and access has been implemented using MOLGENIS open source platform for scientific data that enables flexible data upload, management and querying, including sufficiently rich metadata and interfaces for machine processing and custom (R statistics) visualization for human processing (see http://molgenis.org). Also summaries of the study have been submitted to BBMRI central catalogues https://catalogue.bbmri.nl (Netherlands) and http://www.bbmri-eric.eu/news-events/bbmri-eric-directory-2-0/ (EU).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

Since no the proportion of genetic variation is unknown sample size calculation was not possible. During a previous pilot study using 79 samples multiple genome wide significant QTL’s were found indicating that significant effects can be observed even with limited sample size. In the current analysis we utilized a dataset of ~ 500 individuals for increased power, which allowed us to observe more significant associations. With this we are the largest study to date on cytokine production.

**Data exclusions**

For several of the datasets samples where excluded during the QC process. For genotyping several samples where excluded due to being ethnic outliers. Individuals with severe disease or under heavy medication were excluded from the entire analysis as well.

**Replication**

Most of the experiments where replicated using statistical re-sampling methods to ensure the reliability of the models produced. The prediction models for cytokine response were applied in an independent cohort and several of the tested models could be validated. In addition, we performed several in vitro experiments testing several associations which were found. These experiments validated our hypotheses.

**Randomization**

Where applicable, samples (individuals) where divided into testing an training groups randomly multiple times to ensure the quality of the models produced.

**Blinding**

Blinding was not applicable to the current study since no group allocation was performed. Immune variation was studied in a population based cohort where individuals were not allocated into groups during data collection.

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| [x] Unique biological materials | [x] ChIP-seq |
| [x] Antibodies                  | [x] Flow cytometry |
| [x] Eukaryotic cell lines       | [x] MRI-based neuroimaging |
| [x] Palaeontology               |         |
| [x] Animals and other organisms |         |
| [x] Human research participants |         |
Human research participants

Policy information about studies involving human research participants

Population characteristics
The main analyses were performed in the 500FG cohort, which is part of the Human Functional Genomics Project. This cohort consists of 534 healthy individuals (237 males and 296 females) of Caucasian origin. Volunteers range from 18 to 75 years of age, with the majority (421 individuals) being 30 years or younger. BMI is within normal limits (15 to 35) with the majority (380 individuals) having a BMI between 20 and 25. Of these 534 original volunteers, 45 were excluded based on genetic background and questionnaire results (medication usage, chronic disease) leaving 489 individuals.

Recruitment
Recruitment of samples for 500FG was done on a volunteer basis. The final selection of volunteers included a bias towards younger individuals. This has a potential impact on the generality of the results observed i.e. results observed might not be directly applicable in an older age group.