Impact of rare and common genetic variation in the Interleukin-1 pathway on human cytokine responses

Rosanne C. van Deuren[1,2,3], Peer Arts[2,4], Giulio Cavalli[1,5,6], Martin Jaeger[1,3], Marloes Steehouwer[2], Maartje van de Vorst[2], Christian Gilissen[2,3], Leo A.B. Joosten[1,3,7], Charles A. Dinarello[1,6], Musa M. Mhlanga[8,9], Vinod Kumar[1,10], Mihai G. Netea[1,3,11], Frank L. van de Veerdonk[1,3], Alexander Hoischen[1,2,3]

1. Department of Internal Medicine, Radboud University Medical Center, Nijmegen, the Netherlands
2. Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands
3. Radboud Institute of Molecular Life Sciences (RIMLS), Radboud University Medical Center, Nijmegen, the Netherlands
4. Department of Genetics and Molecular Pathology, Centre for Cancer Biology, SA Pathology and the University of South Australia, Adelaide, South Australia, Australia
5. Unit of Immunology, Rheumatology, Allergy and Rare Diseases, IRCCS San Raffaele Hospital and Vita-Salute San Raffaele University, Milan, Italy
6. Department of Medicine, University of Colorado, Aurora, Colorado, USA
7. Department of Medical Genetics, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
8. Division of Chemical Systems & Synthetic Biology, Institute for Infectious Disease & Molecular Medicine (IDM), Department of Integrative Biological & Medical Sciences, University of Cape Town, Cape Town, South Africa
9. Faculty of Health Sciences, Department of Integrative Biomedical Sciences, University of Cape Town, Cape Town, South Africa
10. University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, The Netherlands
11. Department for Genomics & Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany

Corresponding author: Dr. Alexander Hoischen; Alexander.Hoischen@radboudumc.nl

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ABSTRACT

Interleukin(IL)-1 signaling is of major importance in human innate cytokine responses. Common variants in related genes have been linked to various inflammation-mediated diseases and stimulation-induced cytokine responses, but the role of rare variants remains to be elucidated.

In this study, we characterize the role of rare and common genetic variation, as identified by molecular inversion probe-based sequencing, in 48 genes related to the IL-1 pathway. We examined the inter-individual variability in *in vitro* stimulation-specific human cytokine responses from 463 healthy individuals of the Human Functional Genomics Project and assessed the role of rare and common genetic variants, separately and combined, by means of the Sequence Kernel Association Test.

We identified strong associations for rare genetic variants in *NCF4* ($_{adj}P=7.2E^{-05}$) and *CASP1* ($_{adj}P=3.0E^{-05}$) with IL-6 production in response to PHA and LPS stimulation, respectively. In addition, common variants in *IL36A* and *IL38* were associated to both *C. albicans*-induced IL-1β (*IL36A* $_{adj}P=0.0442$; *IL38* $_{adj}P=0.0092$) and IL-6 production (*IL36A* $_{adj}P=0.0037$; *IL38* $_{adj}P=0.0082$), an effect that was stronger at the subpathway level both for IL-1β ($_{adj}P=0.0017$) and IL-6 ($_{adj}P=1.8E^{-04}$). The common variant signature for the IL-1β and IL-6 response to *C. albicans* was confirmed by an association with all anti-inflammatory genes ($_{adj}P=1.87E^{-03}$ and $_{adj}P=5.75E^{-04}$), and we validated this finding for non-coding common variants. Lastly, we identified a burden of rare variants in pro-inflammatory genes and LPS-induced IL-6 production ($_{adj}P=2.42E^{-04}$), and a new role for anti-inflammatory rare variants on *S. aureus*-stimulated IL-6 production ($_{adj}P=6.71E^{-03}$).

In conclusion, we show that both common and rare genetic variation in genes of the IL-1 pathway, separately and combined, differentially influence *in vitro* cytokine responses to various stimuli in healthy individuals. This study therefore provides insight into potential mechanisms that are translatable into new hypothesis-driven characterization of common and rare variant involvement in a wide variety of inflammatory and immunological mechanisms and diseases.

INTRODUCTION

The innate immune system is our first line of defense against invading pathogens such as viruses, bacteria, fungi and parasites. It consists of a variety of cell populations (*e.g.* myeloid cells, natural killer cells, innate lymphocytes) and soluble mediators (*e.g.* complement), each fulfilling a well-defined function. Collectively, these components shape innate immune responses. The induction of inflammation in response to *e.g.* infection is a naturally occurring and necessary process. Notwithstanding, both stimulatory and inhibitory mechanisms are required to induce effective elimination of pathogens, as well as to limit collateral damage to the tissues, *i.e.* prevent auto-inflammation[**1**].

The Interleukin-1 (IL-1) family of cytokines and receptors plays a major role in the induction and regulation of host defense and inflammation[**2**]. The IL-1 family comprises pro-inflammatory cytokines (*e.g.* IL-1α/β, IL-36α/β/γ), anti-inflammatory cytokines (*e.g.* IL-37, IL-38), activating receptors (*e.g.* IL1-R1, IL-36R), decoy receptors (*e.g.* IL-1R2, IL-18BP), and additional regulators, kinases and phosphatases that altogether are responsible for the IL-1-mediated response[**3**]. Next to core IL-1 family effectors, members of the inflammasome and autophagy pathway are important contributors to the regulation of IL-1-induced inflammation. For instance, activation of the inflammasome allows for cleavage and activation of caspase-1, with subsequent activation and release of pro-inflammatory cytokines IL-1β and IL-18. Conversely, autophagy is an important process involved in cell homeostasis, but is also able to directly inhibit the inflammatory response by removing inflammasome components and damaged mitochondria[**4**].

Defects in IL-1 pathway signaling and its specific members have been linked to various inflammation-mediated diseases[**2, 5**]. Pro-inflammatory members of the IL-1 family, *e.g.* IL-1β and IL-18, play an important role in a variety of (auto-)inflammatory or immune diseases. For instance, a loss of balance in processing and secretion of IL-1β is an important feature of chronic inflammatory conditions like gout, systemic-onset juvenile idiopathic arthritis (sJIA), adult-onset Still's disease (AoSD) and osteoarthritis[**5**]. Moreover, IL-18 plasma concentrations are moderately elevated in systemic lupus erythematodes (SLE) and in rheumatoid arthritis (RA), while being severely increased in patients with macrophage activation syndrome (MAS)[**5**]. Circulating concentrations of anti-inflammatory members of the IL-1 family are characteristically increased in numerous inflammatory conditions. Specifically, plasma concentrations of IL-1Ra, the anti-inflammatory competitor of IL-1α and IL-1β encoded by the *IL1RN* gene, are systemically increased in *i.e.* sepsis, Crohn's disease and ulcerative colitis, and locally increased in the joints from patients with RA and osteoarthritis[**6**]. The IL-1 type 2 receptor, a decoy receptor that binds IL-1 but does not induce an intracellular pro-inflammatory signal, has also been found increased in sepsis and has potential as a candidate biomarker in patients with acute respiratory distress syndrome[**6**].

For a number of (auto-)inflammatory diseases, the clinical presentation clearly points towards dysregulated activity of the IL-1 pathway. A targeted search based on clinical presentation for genetic defects in genes related to the IL-1 pathway, has therefore been proven most useful in patients with CAPS (cryopyrin associated periodic syndromes) and DIRA (deficiency of IL-1 receptor antagonist) with the identification of mutations in *NLRP3* and *IL1RN* respectively[**5, 7**]. On the other hand, even though AoSD, Behcet's and Schnitzler disease share clinical similarities with CAPS, no single causal genetic defect has been identified to date despite the fact that subsets of patients have presented with

mutations in related genes indicating that the likely genetic basis is close[**7, 8**]. In addition to rare variants, a number of Genome Wide Association Studies (GWAS) have identified common variants in the IL-1 pathway to be associated with inflammatory diseases and stimulation-induced cytokine responses[**9-12**]. However, most studies so far have investigated rare and common variants separately. Considering increasing evidence that variability in phenotypic presentation can be caused by an interplay between variants of variable frequencies[**13-15**], or aggregation of genetic variants over genes underlying dysregulated biological mechanisms[**16**], or even over genes that are more distantly involved[**17**], we hypothesize that studies on the genetic basis of inflammatory diseases might also benefit from this concept.

Immune responses are highly variable between individuals, and the broad spectrum of this inter-individual variability ranges from healthy, *e.g.* protection against infection or trauma by inflammatory responses, to diseased individuals, *e.g.* sepsis or auto-inflammatory syndromes caused by excessive inflammation. For this reason, in the past few decades various studies have focused on determining the genetic variation that contributes to the inter-individual variability in innate immune responses[**18-22**]. In summary, these studies show the separate and shared contribution of host and environmental factors to an immunological response after a specific stimulus. Despite these efforts, a considerable percentage of immune response variation between individuals remains unexplained. One important shortcoming is that most studies to date have focused on common genetic variants, while the impact of rare or private variants remains poorly or not at all understood. With recent advancements in sequencing technologies, the ability to study the role of rare variants has remarkably improved, and its value has been proven in several studies. For instance, increasing evidence is showing that the combined effect of common and rare variants could partially explain the missing heritability problem in complex diseases[**13-15**]. The relatively small-to-moderate effects of common variants can be significantly modified by the presence or absence of (multiple) rare variants[**23**]. Molecular Inversion Probe (MIP) based re-sequencing is a targeted sequencing technology that can identify common, low-frequency, rare and even private variants within a region of interest, and as such allows to cost-effectively study intermediate sized gene panels (*e.g.* 50 genes) in relatively large cohorts[**24-27**].

Considering the importance of the IL-1 pathway for inflammation and innate immune responses, we aimed to identify and characterize rare and common genetic variants in 48 genes related to the IL-1 pathway mediated immune response, and determine their impact on the inter-individual variability of cytokine responses in healthy individuals. A complete overview of the study workflow can be found in **Figure 1**.

RESULTS

Study Cohort

Here, we studied healthy individuals from the Human Functional Genomics Project (FG500-cohort, http://www.humanfunctionalgenomics.org/site/), by making use of the publicly available demographic data and stimuli-specific *in vitro* cytokine measurements (https://hfgp.bbmri.nl/). The gender distribution over 463 included individuals for analysis shows a mild overrepresentation of females as compared to males (Male n=201, Female n=262), whereas the mean- and median age distribution for these groups separately is comparable (**Supplementary Figure S1A**).

In vitro IL-1β and IL-6 cytokine production in whole blood in response to stimulation with either 100ng/mL Lipopolysaccharide (LPS), 10µg/mL Phytohemagglutinin (PHA), heat-killed *Candida albicans* 10^6CFU/mL (*C. albicans*) and $1x10^6$/mL *Staphylococcus aureus* (*S. aureus*), were likewise evenly distributed between females and males (**Supplementary Figure S1B**), and were log-transformed prior to analysis. Based on the above-mentioned distributions in combination with the fact that previous research has shown that age and gender can influence cytokine responses[**18-21**], both variables were included as covariates in our analyses.

Sequencing

Molecular Inversion Probe (MIP)-based re-sequencing of all coding exons of the 48 genes in our IL-1 pathway MIP-panel generated sequencing data from 520 healthy individuals (for all MIPs see **Supplementary Table S1**). Overlapping the sequencing data with the available immunophenotyping data we managed to obtain complete datasets from 463 individuals for analysis. The average coverage depth for these 463 individuals over all MIPs was 830x (**Supplementary Figure S2**). Five genes in our panel (*SIGIRR*, *PYCARD*, *CYBA*, *RAC2* and *MAP1LC3A*) were unfavorably covered (<100x average coverage of the entire coding part of the gene) for more than half of the samples, and one gene (*NCF1*) lost all coverage in our extensive quality filtering due to homology regions, and was therefore excluded from subsequent analysis (**Supplementary Figure S2**). Based on gene-encoding protein function and the immunological cascade in which they are activated, these 48 genes were classified prior to the analysis into 1) six subpathway groups: IL-1 subpathway, IL-18 subpathway, IL-30s subpathway, Inflammasome, ROS-production and Autophagy; and 2) two inflammatory-phenotype groups: pro-inflammatory, and anti-inflammatory (**Supplementary Table S2**).

Overall, we identified 201 non-synonymous variants in the coding exon regions, out of which 35 were common and 166 were rare (based on cohort allele frequencies (AFs) using a threshold of ≥ 5% for common variants). Furthermore, out of 166 rare variants we identified 18 variants to be novel, *i.e.* never observed before in public databases. For a complete variant list see **Supplementary Table S3**.

Variant Analysis

The role of rare and common variants on stimuli-specific cytokine responses was assessed by a Rare Variant Burden Analysis (RVBA) using the Sequence Kernel Association Test (SKAT)[**15, 28, 29**]. The main issue with rare variant association testing lies in the low-frequency of rare variants, and thus appropriate grouping is required. We performed the SKAT using three different grouping strategies (**Figure 1D.c** and **Supplementary Table S2**): I) **gene-level**, where all variants within the coding region of each

gene were considered; II) **subpathway-level**, where all coding variants within genes that belong to the corresponding subpathway were considered; and III) **inflammatory-phenotype level**, where genes were classified as either pro-inflammatory or anti-inflammatory based on encoded protein function, and all variants from genes in either group were considered. Each level was assessed for the role of genetic variants on stimuli-specific IL-1β and IL-6 cytokine production, through the full spectrum of AFs (from rare- to common (AF ≥ 5%) variants separately and combined) by performing four different tests (**Figure 1E**): i.SKAToC; testing only common variants, ii.SKAToR; testing only rare variants, iii.SKATjoint; testing common and rare variants jointly by using a method that considers common variant effects as covariates in the rare variant test. To verify potentially missed strong unidirectional signals, the rare variants were additionally subjected to an adapted algorithm that uses a linear combination of the SKAT and Burden Test, the SKAT-O (iv.SKATO). Output from all SKATs performed in this study can be found in **Supplementary Table S4**.

Association landscapes show similarities and differences in IL-1ß and IL-6 response

We created holistic heatmap overviews termed 'association landscapes', for the purpose of summarizing rare- and common variant associations, both on the gene and subpathway level, in an organized fashion. **Figure 2** shows these landscapes of gene- and subpathway based associations for IL-1ß (**2A**) and IL-6 (**2B**) production by whole blood. **Figure 3** shows the inflammatory-phenotype based associations for IL-1ß (**3A**) and IL-6 (**3B**) production by whole blood in classic rectangular heatmaps.

The **gene-level** analysis revealed significant associations between rare genetic variants in *NCF4* and both IL-1ß and IL-6 cytokine production in response to PHA stimulation (SKAToR $_{adj}$P-value=0.0126 and 7.2E^{-05} respectively), that sustained when common variants were considered as covariates (SKATjoint $_{adj}$P-value=0.0185 and 1.3E^{-04} for IL-1ß and IL-6 respectively). Another strong rare variant signal was observed between *CASP1* rare variants and LPS-induced IL-6 cytokine production (SKAToR $_{adj}$P-value=3.0E^{-05}). Next to this, common variants in *IL36A* and *IL38* were significantly associated to both IL-1ß and IL-6 cytokine production after *C. albicans* stimulation (*IL36A* SKAToC $_{adj}$P-value=0.0442 and 0.0037; *IL38* SKAToC $_{adj}$P-value=0.0092 and 0.0082). These associations substantially weakened in the joint analysis of common and rare variants, with the exception of the *IL36A* association with IL-6 that remained comparable to the rare variant result (SKATjoint $_{adj}$P-value=0.0070).

The **subpathway-level** analysis identified a significant association between rare genetic variants of IL-1 subpathway genes combined and LPS-induced IL-1ß cytokine production (SKAToR $_{adj}$P-value=0.0030), that maintained in the joint analysis with only a minor decrease in significance (SKATjoint $_{adj}$P-value=0.0056). Specific to IL-6, we identified associations between rare variants in ROS-production genes after PHA stimulation (SKAToR $_{adj}$P-value=0.0295), and rare variants in Inflammasome genes combined after LPS stimulation (SKAToR $_{adj}$P-value=3.7E^{-04}), while only the latter remained in the joint analysis (SKATjoint $_{adj}$P-value=4.7E^{-04}). Finally, common variants in IL-30s subpathway genes were significantly associated with both IL-1ß and IL-6 cytokine production in response to *C. albicans* stimulation (SKAToC $_{adj}$P-value=0.0017 and 1.8E^{-04} respectively), both slightly weaker in the joint analysis.

The **inflammatory-phenotype level** analysis (**Figure 3**), revealed a strong association between rare variants in genes with pro-inflammatory effects and LPS-induced IL-6 cytokine production (SKAToR $_{adj}$P-value=2.42E^{-04} and SKATO $_{adj}$P-value=1.99E^{-03}), that was recurrent in the joint analysis (SKATjoint $_{adj}$P-value=3.60E^{-04}). On the other hand, rare

variants in anti-inflammatory genes were significantly associated with IL-6 cytokine production in response to *S. aureus* stimulation (SKATO $_{adj}$P-value=6.71E^{-03}). Moreover, we identified a role for common variants in anti-inflammatory genes in both IL-1ß and IL-6 cytokine production in response to *C. albicans* stimulation (SKAToC $_{adj}$P-value=1.87E^{-03} and 5.75E-^{04} respectively), that in the joint analysis persisted for IL-1ß (SKATjoint $_{adj}$P-value=7.11E^{-03}), whereas in for IL-6 most of the signal was lost (SKATjoint $_{adj}$P-value=0.0240). Finally, unique to *C. albicans*-induced IL-6 production, was an association with common variants in pro-inflammatory genes (SKAToC $_{adj}$P-value=0.0153).

Gene-level rare variant associations of cytokine outliers imply cytokine-stimulus specific features

The associations presented thus far were produced by using stimulus-specific cytokine profiles of all individuals as continuous measurements. In order to uncover rare variant gene associations that may reflect strong stimulus-specific mechanisms, we supplemented these continuous trait associations (CT), with extreme binary trait associations (BT). The BT associations were conducted by categorizing individuals into two groups, based on 1% highest and lowest cytokine production respectively, in response to a certain *in vitro* stimulus, from here on referred to as top-producers ($_{TOP}$BT) and low-producers ($_{LOW}$BT). Identifying recurrent rare variant associations (that is, nominally significant in CT and either $_{TOP}$BT or $_{LOW}$BT), allowed us to give meaning in terms of direction of the effect of our continuous association results. **Figure 4** shows recurrent gene-level rare variant association (SKAToR) P-values, separately for IL-1ß (**A**) and IL-6 (**B**).

The analysis of extreme cytokine producers was consistent with our identified continuous association result between rare variants in *NCF4* and PHA-induced IL-1ß and IL-6 cytokine production, by the identification of a recurrent signal in the low-producers (IL-1ß $_{LOW}$BT P-value=2.18E^{-04}; IL-6 $_{LOW}$BT P-value=5.94E^{-05}), along with the association between *CASP1* and IL-6 low-producers after LPS stimulation ($_{LOW}$BT P-value=0.0299). In addition to that, we identified three recurrent associations in IL-1ß top-producers: rare variants in *IL1R2* after LPS- (P-value=0.0429) and PHA stimulation (P-value=0.0493); *IL18BP* rare variants after *S. aureus* stimulation (P-value=0.0038); and four recurrent associations in LPS-induced IL-1ß low-producers - rare variants in *NCF4* (P-value=8.27E^{-03}), *IL1R1* (P-value=0.0194), *IL1RL2* (P-value=8.54E^{-04}) and *IL36G* (P-value=3.03E^{-03}) (**Figure 4A**). In the case of IL-6 (**Figure 4B**) we detected one additional recurrent signal, that is *IL33* rare variants and top-producers after *C. albicans* stimulation (P-value=0.0143).

Immunological response to C. albicans reflects a common variant signature

Our results of the SKAToC show strong common coding variant set signals over various levels of magnitude both on IL-1ß and IL-6 cytokine production after *C. albicans* stimulation, reflecting a common variant signature in this immunological response. For the separate variants in *IL36A* (rs895497) and *IL38* (rs6761276 and rs6743376), we observed that the alternative allele presented with 1) a higher frequency as compared to the ancestral allele, and 2) a higher cytokine production (residual, after correcting for co-variates age and gender). **Figure 5A** shows that for each of these variants the IL-1ß and IL-6 *in vitro* cytokine production in response to *C. albicans* stimulation decreases over the genotype categories. Complementary Wilcoxon-rank-sum tests confirmed that for all three variants homozygous reference versus homozygous alternative and heterozygous versus homozygous alternative, was significantly associated with higher IL-1ß and IL-6 cytokine production, and homozygous reference versus heterozygous only for *IL38* rs6743376 (**Figure 5A**).

While the rest of our study focused on coding variants, *i.e.* variants that likely have a direct effect on protein function, we additionally aimed to get insight into the impact of non-coding common variants. Indeed, accumulating evidence highlights a role for common non-coding genetic variation in human health[**30, 31**], in inflammatory responses[**32, 33**], and even specifically in innate immune responses[**34-36**]. Therefore, for the purpose of replicating our coding common variant signals, we repeated our significantly associated common variant sets in this study with previously published genotyping data from the same (FG500) cohort containing coding and non-coding common genome-wide genetic variation (https://hfgp.bbmri.nl/). All common variants (cohort AF>0.05) within a set were pruned for Linkage Disequilibrium (LD), using within cohort R^2 metrics. **Figure 5B** shows that by subjecting these expanded sets to the same SKAT, we identified associations between *C. albicans*-induced IL-6 production and non-coding common variants in *IL36A* (P-value=0.049), *IL38* (P-value=0.007) and pro-inflammatory genes (P-value=0.019), and between *C. albicans*-induced IL-1ß production and non-coding common variants in *IL38* (P-value=0.046). We visualized these associations by calculating an allelic score, with single SNP directions and MAF-based variant weights incorporated, for all significant sets in **5B**. In **Figure 5C** the weighted directional allelic score is displayed in correlation with cytokine production, demonstrating that for each set there is an increasing cytokine production with increasing score, highest for *IL38* set with a correlation (R^2) of 0.025. Finally, to evaluate the individual contribution of non-coding common variants in a set, we organized single SNP effect estimates based on their direction and significance (**Supplementary Figure S3**). A more in-depth follow up on *IL36A* set non-coding SNP rs1562305, revealed its non-coding activity by a combination of an interaction with *IL36G* and *IL36A* based on K562 Hi-C data and Pol II-associated chromosomal contacts are observed in the regions surrounding rs1562305 and *IL36A* (**Supplementary Figure S4A and B**). Moreover, rs1562305 falls within a region that is bound by several transcription factors, including *HNF4A*, that has been associated with ulcerative colitis and diabetes mellitus type II (**Supplementary Figure S4C**)[**37**].

DISCUSSION

In this study we identify and characterize rare and common genetic variation in genes related to the IL-1 pathway, and determine their impact on the inter-individual variability of stimulus-specific *in vitro* cytokine responses, measured in whole blood from healthy individuals. In addition, we assess the relative contribution of rare as compared to common variants, as well as the joint effect of rare and common variants, by employing various grouping strategies.

Over the past decades, multiple studies have identified a role for common genetic variation on cytokine level and response, however a significant proportion of inter-individual variability remains to be determined[**18-21**]. Common and rare variants have mostly been studied separately, and the differentiation between them is an arbitrary decision. Considering increasing evidence that specific combinations of variants with variable frequencies accounts for variability in phenotypic presentation[**13-15**], in particular for a combination of phenotypic characteristics that do not fit one specific clinical diagnosis[**38**], we hypothesized this concept might also apply to the inter-individual variability in cytokine responses. As such, this study aimed to provide a more holistic view, that considers the interplay of variants of different allele frequencies. For this purpose, we sequenced the coding regions of 48 genes related to the IL-1 pathway in almost 500 healthy individuals, and assessed *in vitro* IL-1ß and IL-6 production by whole blood in response to LPS, PHA, *C. albicans* and *S. aureus.* By means of the Sequence Kernel Association Test (SKAT), we tested for association between cytokine production and only common- (SKAToC), only rare- (SKAToR), and common and rare variants combined (SKATjoint), over various levels of grouping strategies; gene-, subpathway, and inflammatory-phenotype groups.

The strongest rare variant association detected in this study was between *CASP1* and LPS-induced IL-6 cytokine production. CASP-1 protein, encoded by *CASP1* gene, is responsible for cleavage of the inactive mediators IL-1ß, IL-18 and IL-33 into their active form. The association in this study was based on five rare variants: two private variants (one new to public databases); two variants identified in two individuals in heterozygous state, and one variant identified in five individuals in homozygous state (see **Supplementary Table S3**). The fact that we observed a burden of rare variants not with IL-1ß but with IL-6 production, may suggest an unknown effect of CASP-1 on IL-6 production and/or release, although this is a speculation and remains to be demonstrated in future studies. Alternatively, immune responses are highly dynamic and as such can be influenced differentially by genetics over time[**39**], and since we used cytokine measurements after 48 hours of stimulation, it could also reflect an unnoticed effect of these variants on preceding IL-1ß production, that subsequently influence the induction of IL-6.

We detected an almost equally strong rare variant burden between *NCF4* and both IL-1ß and IL-6 production in response to PHA stimulation. This association was based on three private rare variants, one of which is new to public databases. Two variants are located in the canonical splice-acceptor sites, thereby possibly affecting the splicing of this gene, which most likely leads to loss-of-function variants with reduced RNA and possibly protein levels as a consequence. The *NCF4* gene encodes the NCF4 protein which is part of the cytoplasmic unit of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase enzyme system involved in phagocytosis[**40**]. Variation in NADPH related genes are clinically relevant for human immunity as, rare mutations have been linked to chronic granulomatous disease (a primary immunodeficiency), and common variants have been

associated with immune related diseases like Crohn's disease and systemic lupus erythematodes (SLE)[**41, 42**]. Knock-out of *Ncf4* in mice results in a defective NADPH-complex, with as a consequence reduced ROS-production and inefficient phagocytosis, ultimately leading to increased cytokine production[**43, 44**]. The association detected in this study between *NCF4* rare variants and decreased cytokine production therefore suggests a gain-of-function effect on the NADPH-complex. However, considering the splice-affecting characteristics of two of these rare variants, the resulting effect on the NADPH oxidase system remains inconclusive and requires further investigation.

In addition, our study revealed an association between rare variants in anti-inflammatory genes and *S. aureus*-induced IL-6 production. The unidirectional characteristic of this was reflected by the observation that in whole blood from individuals carrying a rare variant more IL-6 cytokine was produced in response to *S. aureus*, as confirmed by Wilcoxon rank-sum P-value=0.0054 (**Figure 3C**). Remarkably, more than half of the anti-inflammatory genes are autophagy genes, which is in line with the notion that defective autophagy results in increased cytokine production, with increased inflammatory disease severity, like colitis and CGD, as a consequence[**43, 45**].

An intrinsic issue with rare variants is their low-frequency, resulting in limited power for association testing[**46**]. Combining multiple rare variants followed by appropriate combination tests can improve power, but requires prior knowledge on the biological effects of the genes studied. Our IL-1 subpathway rare variant association with LPS-induced IL-1ß production confirms that appropriate grouping produces a significant association (SKAToR $_{adj}$P-value=0.003), even though the individual genes did not (**Figure 2A**). The most likely reason for this is insufficient power, and indeed a thorough inspection of the raw data disclosed significant underlying gene-level association P-values that did not survive the Bonferroni-adjustment. The subpathway level association between common variants in genes of the IL-30s subpathway and IL-6 production in response to *C. albicans* illustrates that even for common variants appropriate grouping can increase power. For instance, the IL-30s subpathway association with *C. albicans* stimulated IL-6 cytokine we detected, was more significant than could be explained by the gene-level associations with *IL36A* and *IL38*. Even though common variants in other members of the respective subpathway (*i.e. SIGIRR* and *IL37*) individually were not associated, most likely they do contribute to the subpathway signal. The *C. albicans*-specific common variant signature identified in this study by combining multiple common variants in a set over several levels of magnitude, additionally supports Smeekens et al.[**47**], where they identified the innate immune pathway to be induced by *C. albicans*. Next to highlighting the role for coding common variants, our validation with non-coding common variants in the same cohort supports the importance of considering interplay between multiple common variants, and additionally substantiates the urge for studying the impact of epigenetic regulation in immunity[**48**]. Moreover, we identified a potential epigenetic mechanism that may allow insights into the gene regulation by non-coding variants, for rs1562305 on *IL36A* and *IL36G* gene expression levels (**Supplementary Figure S4**).

Another method to increase power in rare variant association studies, is to use extreme phenotypes for the purpose of enriching rare variants with strong(er) effects in outlier individuals[**49, 50**]. In this study, we used 1% extreme cytokine producers in a binary association and overlapped the results with our continuous tests, for the purpose of 1) characterization of stimulus-specific mechanisms, and 2) providing an indication of direction. Our strongest continuous rare variant associations (*CASP1* with LPS-induced IL-6 cytokine

and *NCF4* with PHA-induced IL-1ß and IL-6 cytokine), reemerged in low-responders. The fact that these associations were identified both in our continuous and binary analyses, is in line with the finding that extreme phenotyping can enrich for the presence of rare causal variants[**49, 50**]. Remarkably, our *NCF4* private variant carriers were restricted to the 1% lowest PHA-induced IL-6 cytokine response, substantiating that most extreme variants (in terms of lowest frequency) can have most extreme effects on the phenotype level[**51-54**]. In addition, we identified recurrent associations between LPS-induced IL-1ß cytokine production and five genes of the IL-1 subpathway, substantiating the subpathway level association and at the same time reflecting lack of power in the identification of individual gene associations. Interestingly, we identified an association between *S. aureus*-induced IL-1ß cytokine production and rare variants in *IL18BP*, recurrent in the top-responders. The encoded protein (IL-18BP) exerts major anti-inflammatory effects by inhibiting IL-18 signaling[**55**], and previous studies have shown that IL-18BP levels are negatively correlated with cytokine production by lymphocytes[**18**]. The identified burden in top-responders here, suggests that rare variants in *IL18BP* negatively affect IL-18BP levels, allowing for higher *S. aureus*-induced cytokine production. The role of IL-18/IL-18BP pathway in cytokine responses to *Staphylococci* therefore needs further investigation, as it has the potential to provide important new insights in severe diseases caused by this pathogenic microorganism (*e.g.* sepsis).

Our study cohort is one of the largest to date in which extensive immunophenotyping experiments have been performed[**18, 19, 21**]. The associations described here are based on cytokine production by whole blood, as it is most comparable to the *in vivo* situation and therefore most representative for physiologic immune responses. Furthermore, the investigated stimuli were chosen as representatives for an array of microbial infections, specifically: LPS is expressed on the bacterial cell wall of Gram-negative bacteria; PHA is synthesized by *Bacillus Rhodococcus* and *Pseudomonas species*; and *C. albicans* and *S. aureus* are major invading pathogens representative of fungi and Gram-positive bacteria, respectively. Future efforts investigating a broader array of pathogens, as well as specific contribution of immune cell subtypes, based on the additional publicly available data for this cohort (https://hfgp.bbmri.nl/) is therefore highly recommended. Nevertheless, potential limitations of this study cohort include the relatively small sample size and cohort characteristics (restricted age distribution and residency), and replication in a larger cohort for validation is favorable. Secondly, despite the cost-effectiveness of MIP-sequencing (*e.g.* ± €25,- per sample for the IL-1 panel), larger intronic or non-coding regions are not sequenced and as such escape analysis. The potential of using whole genome sequencing data to investigate the role of rare non-coding genetic variation, thereupon seems promising. Thirdly, the SKAT is powerful, but computes only set-wise association P-values and does not provide single variant effect estimates, neither does it provide direction in terms of positive/negative effects or increased/decreased risk. As we cannot exclude interaction effects between variants in a set, the contribution of single variants to a phenotype is difficult to estimate and as such the clinical applicability remains complex and requires more in-depth follow-up. Lastly, this study was designed for the purpose of discovering correlations between genetic variants and functional cytokine read-outs, but with the prospect of future, in-depth functional follow-up studies. In order to maximize the discovery layer, we Bonferroni-adjusted p-values based on the number of groups tested per stimulus-cytokine combination, frequency and grouping-level, and as such we did not apply post-hoc corrections.

In conclusion, this study shows that common and rare genetic variation in genes of the IL-1 pathway, separately and combined, differentially influence *in vitro* IL-1ß and IL-6 cytokine responses induced by various stimuli. Not only do we add to the knowledge on the role of common variants in the IL-1 pathway, we additionally highlight the important role of rare variants (alone or in combination) on immune response variability. On a broader perspective, this study provides insight into potential mechanisms that are translatable to new hypothesis-driven identification of rare variant involvement in inflammatory and immunological mechanisms and diseases. Over and above, the framework used in this study is expandable to a wide variety of (non-immune) complex phenotypes, and as such can lead the way to new insights and theories for any phenotype of interest, and therefore has the potential to contribute to better understanding of unresolved, complex diseases.

MATERIAL AND METHODS

Study Cohort

Cohort characteristics

The study was conducted using healthy individuals from the Human Functional Genomics Project (500FG-cohort, http://www.humanfunctionalgenomics.org/site/). The entire 500FG-cohort consists of 534 healthy individuals from the Netherlands (296 females and 237 males) with an age range 18-75, from which we were able to obtain DNA from 520 individuals for sequencing. For more details on cohort characteristics see previous publications on the 500FG-cohort[**18, 19, 21**].

Immunophenotyping

In this study we made use of the publicly available extensive immunophenotyping data that was generated as part of the Human Functional Genomics Project (https://hfgp.bbmri.nl/). Specifically, Interleukin-1ß (IL-1ß) and Interleukin-6 (IL-6) production by whole blood (consisting mainly of polymorphonuclear cells (PMNs)) from 471 individuals, stimulated with either lipopolysaccharide (LPS, 100ng/mL), phytohaemagglutinin (PHA, 10µg/mL), heat-killed *Candida albicans* (*C. albicans* 10^6 CFU/mL) or *Staphylococcus aureus* (*S. aureus* 1 x 10^6/mL). A detailed description of these experiments can be found elsewhere[**19**]. In short, blood was drawn from participants and 100 µL of heparin blood was stimulated with 400 µL of stimulus, subsequently incubated for 48 hours at 37°C and 5% CO_2 and supernatants were collected and stored in -20°C until used for ELISA.

Sequencing

MIP-panel design

We sequenced all coding exons of 48 genes of the IL-1 pathway in 520 healthy individuals by Molecular Inversion Probe (MIP)-based re-sequencing. MIPs are a targeted re-sequencing technology, that allows for the identification of both common- and rare genetic variation in regions of interest. A detailed description of MIP-probe design and sequencing methods can be found elsewhere[**24, 27, 56**]. In short, 1285 MIP-probes were designed to cover all coding exons of 48 genes related to the IL-1 pathway and sequencing was performed using the Illumina NextSeq500 system. These 48 IL-1 pathway related genes can be further functionally subclassified into six subpathways that represent a sub-mechanism or immunological cascade in the IL-1-mediated inflammatory response: IL-1 subpathway, IL-18 subpathway, IL-30s subpathway, Inflammasome, (reactive oxygen species) ROS-production, and Autophagy. An additional functional subclassification into two inflammation phenotype groups, was based on the gene-encoded protein function and its pro- or anti-inflammatory effect. A full explanation on the subclassifications can be found in **Supplementary Table S2**.

Data processing

A carefully developed filtering pipeline, based on Sanger Sequencing validations, was applied to ensure high sensitivity and specificity in our final variant set. First, the reads were aligned using BWA-MEM[**57**] and subsequently filtered on Mapping Quality ≥ 60, no soft-clipping, properly paired and not more than five variations from the reference per read, with the exception of multi-basepair insertions and deletions. Variants were then called using

the GATK unified genotyper[**58**], which uses a Bayesian genotype likelihood model to estimate the most likely genotypes. Rare variants (here defined as absent in dbSnp build 150 common[**59**], or defines as rare by our custom annotator as explained below), were further filtered on the QUAL parameter ≥ 1000 in the vcf. Additionally, the percentage of alternative alleles for each variant position using samtools mpileup[**60**], with maximum read depth 10000, no BAQ and a minimal base quality of 30. Homozygous rare variants required an alternative allele percentage of ≥ 90%, heterozygous rare variants required an alternative allele percentage of 25% and < 90%, and rare variants with an alternative allele percentage of < 25% were considered false positive. Samples with an average coverage depth of all MIPs ≥ 100x were included for analysis. The final variant set was annotated using our custom annotator, which makes use of several annotation sources, among others the Variant Effect Predictor (VEP) from Ensembl[**61**], Combined Annotation Dependent Depletion (CADD) score[**62**], and several population based variant databases (*e.g.* dbSnp, ExAc and gnomAD[**52**]) and an "inHouse" database consisting of > 25,000 clinical exomes run at the diagnostic division of the Department of Human Genetics of the Radboudumc. We used within cohort allele frequencies (AFs) to separate rare and common variants, based on a common variant cut-off of ≥ 5%.

Variant Analysis

Continuous Trait

A Rare Variant Burden Analysis (RVBA) was performed on the log-transformed cytokine levels by using the Sequence Kernel Association Test (SKAT)[**15, 28**] in R version 3.5.2. The SKAT is a kernel-based test method, that aggregates weighted individual variant-score test statistics whilst allowing variant-variant interactions and is extremely powerful when a genetic region has both protective and deleterious variants or many non-causal variants[**15, 28, 29**]. The SKAT was performed over three levels of grouping: I) **gene-level**; where all variants within coding exons of a gene region were considered, II) **subpathway-level**; where all variants within genes that belong to the corresponding subpathway were considered, and III) **inflammatory-phenotype level**; where based on encoded protein function genes were classified as either pro-inflammatory or anti-inflammatory and all variants from genes in either groups were considered (**Figure 1D.c**). Furthermore, for each region the 'normal' SKAT was run to determine the effect of only common (i.SKAToC), only rare (iii.SKAToR) and common and rare variants combined (ii.SKATjoint), based on a cohort MAF of 5% as cut-off, using the SKAT_CommonRare function with default weights. However, since the SKAT can be less powerful than burden tests when rare variants in a set are truly causal or influencing the phenotype in the same direction[**29**], we additionally applied the SKAT-O algorithm to the rare variants (SKATO), appropriately weighing the variants with the same weights as described above, and extracted accompanying rho-values to assess the contribution of SKAT versus Burden Test for significant sets reflecting the proportion of bi- and unidirectionality of an association. In the case of rare- and joint tests only output based on > 1 variant was considered, and in the case of joint tests the presence of both rare and common variants in the set was an additional requirement. P-values were Bonferroni-adjusted for each previously defined test separately, based on the number of groups tested. We did not apply additional corrections for the different grouping-levels (*i.e.* gene-level, subpathway-level, inflammatory-phenotype level), nor for the different variant frequency tests (*i.e.* SKAToC, SKATjoint, SKAToR, SKATO).

Binary Trait

In addition to the associations of continuous cytokine levels as described above, we performed a binary association analysis on outlier individuals, here defined as extreme cytokine producers. As research has shown that individuals with outlier expression patterns are likely to be enriched in nearby rare variants[**49, 50, 63, 64**], we hypothesized that outlier individuals in terms of cytokine production could similarly be enriched in rare variants in specific genes, thereby favoring the identification of stimulus-specific mechanisms. For this purpose, we defined for each cytokine-stimulus the 1% extreme cytokine producers (rounded up, so generally ±5 individuals), which categorized the individuals in two groups that were subjected to binary trait association. Specifically, for each cytokine-stimulus combination two tests were performed: 1% highest cytokine producers versus all other individuals ($_{TOP}$BT); and 1% lowest cytokine producers versus all other individuals ($_{LOW}$BT). In two cases, *C. albicans*-induced IL-1ß production low-producers and LPS-induced IL-6 production high-producers, no distinctive categories could be created due to equal cytokine measurements at the 1% cut-off, and as such the groups were extended to 7 and 9 respectively. We followed up on associations based on >1 variant, that were nominally significant (unadjusted P-values) in the continuous associations and recurrent in either $_{TOP}$BT or $_{LOW}$BT.

Follow Up

In order to give meaning to our detected associations, we extracted the residual (corrected for covariates age and gender) cytokine production from the SKAT null-model and correlated those to the genotype categories, where applicable. For all plots, correlations and cytokine levels mentioned from hereon, the cytokine production therefore has been corrected for age and gender. In the case of set-based common variant associations we correlated cytokine production to the three separate genotype categories; homozygous reference, heterozygous, homozygous alternative, whereas in the case of set-based rare variant associations we correlated cytokine production to only two categories; absence or presence of one of the rare variants in the set.

Additionally, considering accumulating evidence for a role of non-coding genetic variation in health and disease[**30, 31**], we followed-up on common variant associations by using the publicly available genotype data from the FG500-cohort, generated with the commercially available SNP chip Illumina HumanOmniExpressExome-8v1.0 (for further details we refer to previously published work[**19, 65**]). We extracted all common variants (based on cohort AF ≥ 5%) within NCBI RefSeq 'Whole Gene' gene regions and extended the start position by 50kB[**66**] for the following sets: *IL36A*, *IL38*, IL-30s subpathway, pro-inflammatory phenotype and anti-inflammatory phenotype. Variant sets were pruned for Linkage Disequilibrium (LD) based within cohort metrics and the commonly used R^2 cut-off of >0.8, by means of the snpStats package in R. The final pruned set of variants, termed tagSnps, were subjected to the same SKAT with default weights, to test for association with continuous IL-1ß (N=428) and IL-6 (N=425) cytokine production. Finally, for the purpose of correlating significant non-coding common variant sets to cytokine levels, we calculated an allelic score for all variants in the set. An allelic score is a way to collapse multidimensional genetic data associated with a risk factor into a single variable[**67**]. We slightly adapted the allelic score calculation to our SKAT-based test results, into a weighted (based on AF-based Beta.Weights function from SKAT package), directional (based on increasing or decreasing cytokine production over the genotype categories) allelic score. Specifically, we inferred the direction of each variant in a set, and combined this with the manually computed variant

weight, by negating the weight only for variants with decreasing cytokine production over the genotype categories. Common variant genotypes were converted to dosages and multiplied by their directional weight, which was summed up to an allelic score per set of variants. The weighted, directional allelic score was plotted versus the cytokine production, and a linear model equation and R^2 metrics were extracted. In addition, to evaluate the individual contribution of non-coding common variants in a set, we computed per SNP linear models using *C. albicans*-induced residual cytokine production as the criterion variable and the SNP in question as predictor variable. The individual SNP effect estimates (or Beta-estimates) were organized by direction and annotated based on their significance. For the purpose of prioritizing one SNP for investigating epigenetic effects we computed the same linear models now using the log-transformed cytokine production as criterion variable and next to the SNP also age and gender as covariates. The predictive capacity of the linear models, as reflected by the model p-value, was used as a measure for impact of a specific SNP on cytokine production and as such prioritized rs1562305 for more in-depth follow-up. Epigenetic effects of rs1562305 were studied using a Hi-C dataset[**68**] and Pol II ChiA-PET libraries for K562 (GSM970213). Transcription Factor ChiP-seq Clusters (338 factors, 130 cell types) were obtained from ENCODE 3 Data version: Encode 3 Nov 2018.

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AUTHOR CONTRIBUTIONS
MGN, FLvdV and AH designed the project. RCvD, MGN, AH conceptualized the experiments and analysis. PA, MJ, MS performed the experiments. RCvD performed data analysis and data visualization. MvdV and CG supported the data analysis. MMM performed additional data analysis. GC, VK, CAD, LABJ, FLvdV and MGN helped with data interpretation. RCvD, MGN and AH wrote the manuscript. All authors contributed to and approved the final manuscript.

CONFLICT OF INTEREST
LABJ reports to be Scientific Advisory Board member of Olatec Therapeutics LLC. CAD serves as chair of SAB of Olatec Therapeutics LLC. All other authors declare that they have no conflict of interest.

DATA AVAILABILITY
All demographic-, immunophenotyping- and genotyping- data from the FG500-cohort used in this study is publicly available on the BBMRI-NL archive (https://hfgp.bbmri.nl/). All variants called in MIP-sequencing data based on the IL-1 panel from the same cohort are published here, as well as all association results.

CODE AVAILABILITY
Code for processing and filtering MIP-based sequencing data are extensively explained in the methods section of this manuscript and will be made available upon reasonable request. The source code from the R packages used in this study are freely available online. Code for processing the output and generating the figures will likewise be made available upon reasonable request.

URLS
Human Functional Genomics Project home site:
http://www.humanfunctionalgenomics.org/site/
Human Functional Genomics Project publicly available data: https://hfgp.bbmri.nl/

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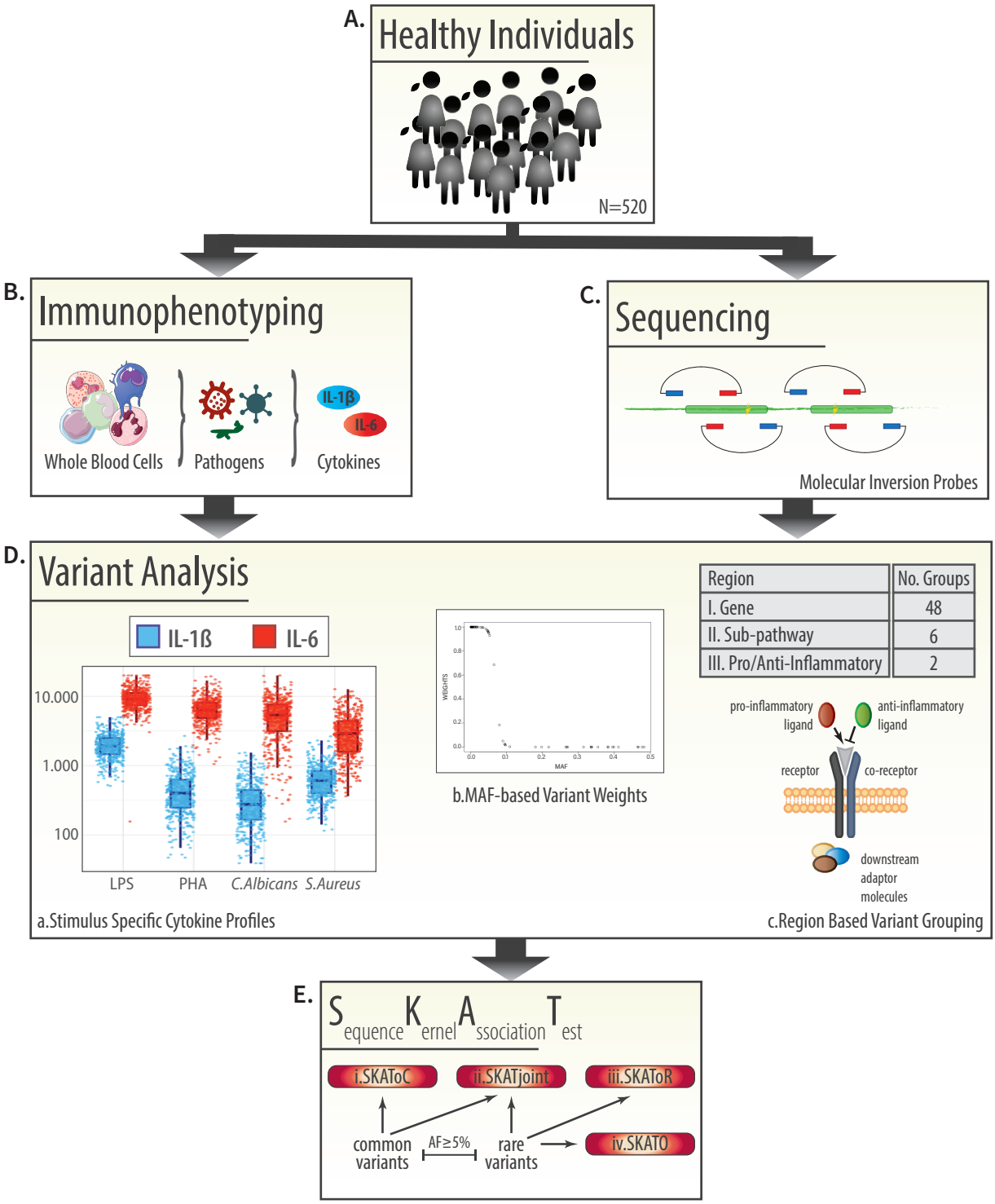


Figure 1. Flowchart of the study workflow method. Figure orientation from top to bottom. Blood was extracted from 520 healthy individuals (A) on which extensive immunophenotyping was performed (B) and simultaneously Moleclar Inversion Probe sequencing data was produced from coding regions of 48 Interleukin-1 pathway genes (C). The resulting cytokine production after stimulation was measured and used for analysis (D-a.Stimulus Specific Cytokine Profiles). Similarly, the identified variants were classified based on their Minor Allele Frequency (MAF), which determined their weighing factor (D-b.MAF-based variant weights). Variants were grouped over three different levels: I.Gene-level; where all variants within coding exons of a gene-region were considered, II.Subpathway-level; where all variants within genes that were classified in the corresponding subpathway were considered, and III.Pro/Anti-inflammatory level; where based on known gene-encoded protein function genes were classified as either pro- or anti-inflammatory and all variants within either pro-inflammatory group or anti-inflammatory group were considered for association testing (D-c.Region Based Variant Grouping). Finally, each of these sets of variants were subjected to four different versions of the Sequence Kernel Association Test (SKAT), based on cohort allele frequency classification: SKAToC; SKAT with only common (AF ≥ 5%) variants (E-i.SKAToC), SKATjoint; SKAT with common and rare variants (E-ii.SKATjoint), SKAToR; SKAT with only rare variants (E-iii.SKAToR), and SKATO; best combination of the SKAT and Burden Test with only rare variants (E-iv.SKATO).

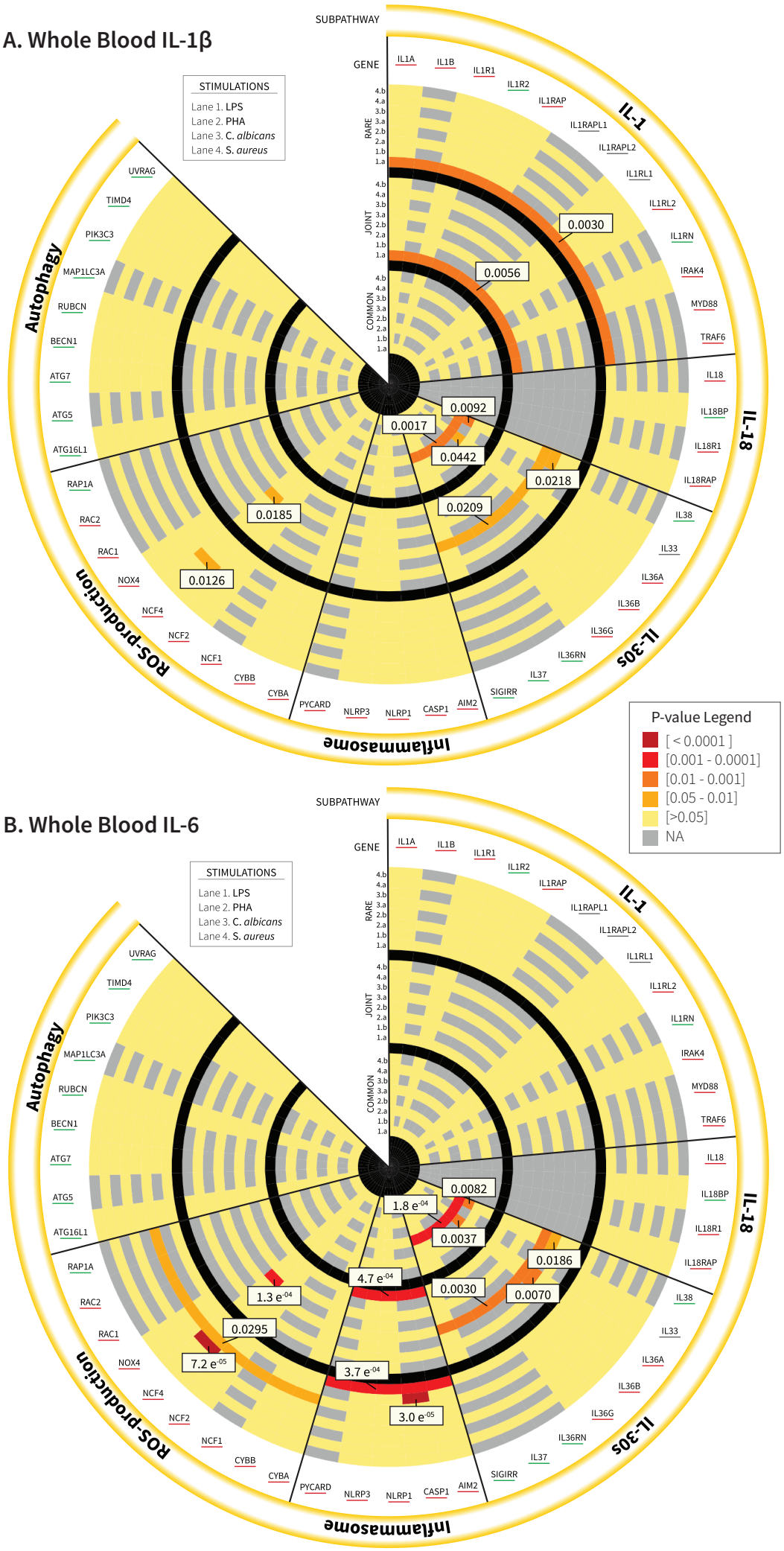


Figure 2. Circular heatmaps of SKAT $_{adj}$P-values. The circular heatmaps consist of three rings separated by black lanes, representing from inner to outer the SKAToC (SKAT with only common (AF ≥ 5%) variants), SKATjoint (SKAT with common and rare variants), and SKAToR (SKAT with only rare variants) $_{adj}$P-values with log-transformed IL-1β and IL-6 cytokine production respectively. Each ring consists of 8 lanes, that represent different stimuli; 1) LPS 100ng/mL, 2) PHA 10µg/mL, 3) *C. albicans* 10^6CFU/mL, 4) *S. aureus* $1x10^6$/mL, with sublane a) showing the subpathway-level result and sublane b) showing the gene-level result. The genes annotated at the surface of the heatmap are underlined with a red, green or grey colour, representing their classification in the inflammatory-phenotype level groups (**Figure 3**).

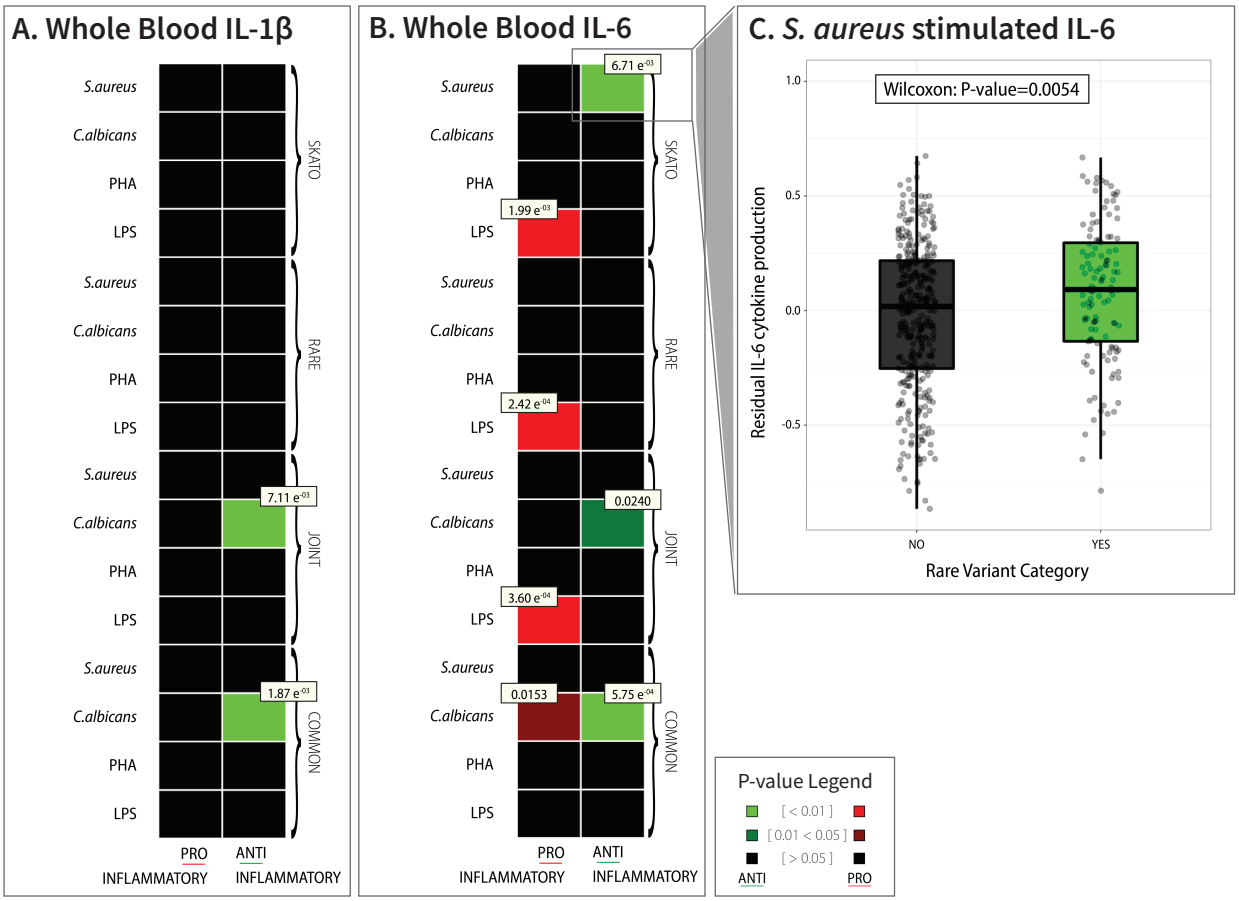


Figure 3. Inflammatory-phenotype level SKAT adjP-values. A heatmap representation of association between inflammatory-phenotype groups and IL-1β (A) and IL-6 (B) cytokine production in response to four different stimuli; LPS 100ng/mL, PHA 10µg/mL, heat-killed *C. albicans* 10^6 CFU/mL, and *S. aureus* $1x10^6$/mL. Variants in genes categorized as pro- or anti-inflammatory were subjected to four different association tests; SKAToC (common (AF ≥ 5%) variants only), SKATjoint (common and rare variants), SKAToR (rare variants only), and SKATO (combination of SKAT and Burden Test with rare variants only). (C) shows a boxplot of *S. aureus* stimulated residual IL-6 cytokine production (corrected for age and gender) over rare variant categories (NO=individuals without rare variant; YES=individuals carrying a rare variant) within genes of the anti-inflammatory phenotype group. Wilcoxon rank-sum test P-value reveals a significant difference between the two categories.

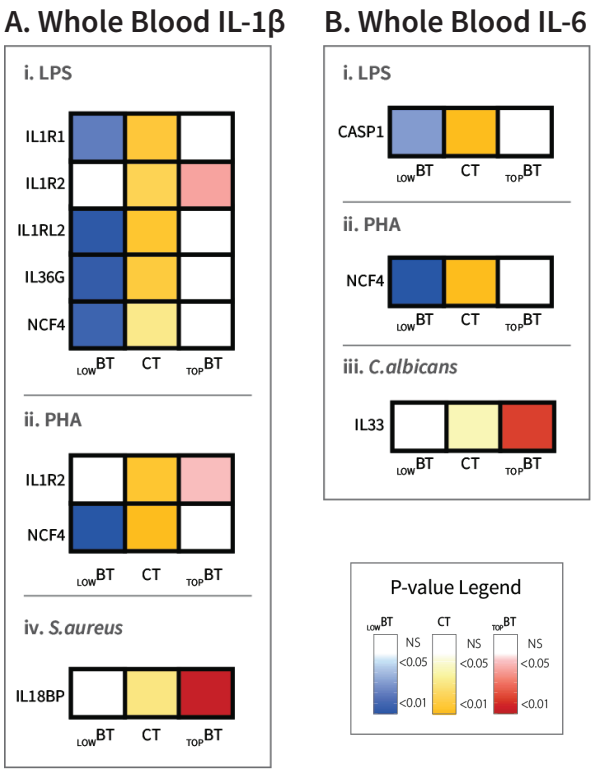


Figure 4. Recurrent gene-level rare variant SKAT P-values in extreme cytokine producers reflect direction of continuous associations and identify distinct cytokine-stimulus specific features. A heatmap representation of all recurrent gene-level rare variant SKAT P-values with IL-1β (A) and IL-6 (B) cytokine production in response to LPS 100ng/mL (i.), PHA 10µg/mL (ii.), *C. albicans* 10^6CFU/mL, and *S. aureus* $1x10^6$/mL stimulation (iv.).

Annotation: $_{LOW}$BT = SKATBinary with lowest 1% producers; CT = SKAT with log-transformed continuous cytokine producers; $_{TOP}$BT = SKATBinary with highest 1% cytokine producers.

A. Coding *IL36A* and *IL38* common variant genotype categories with IL-1β and IL-6

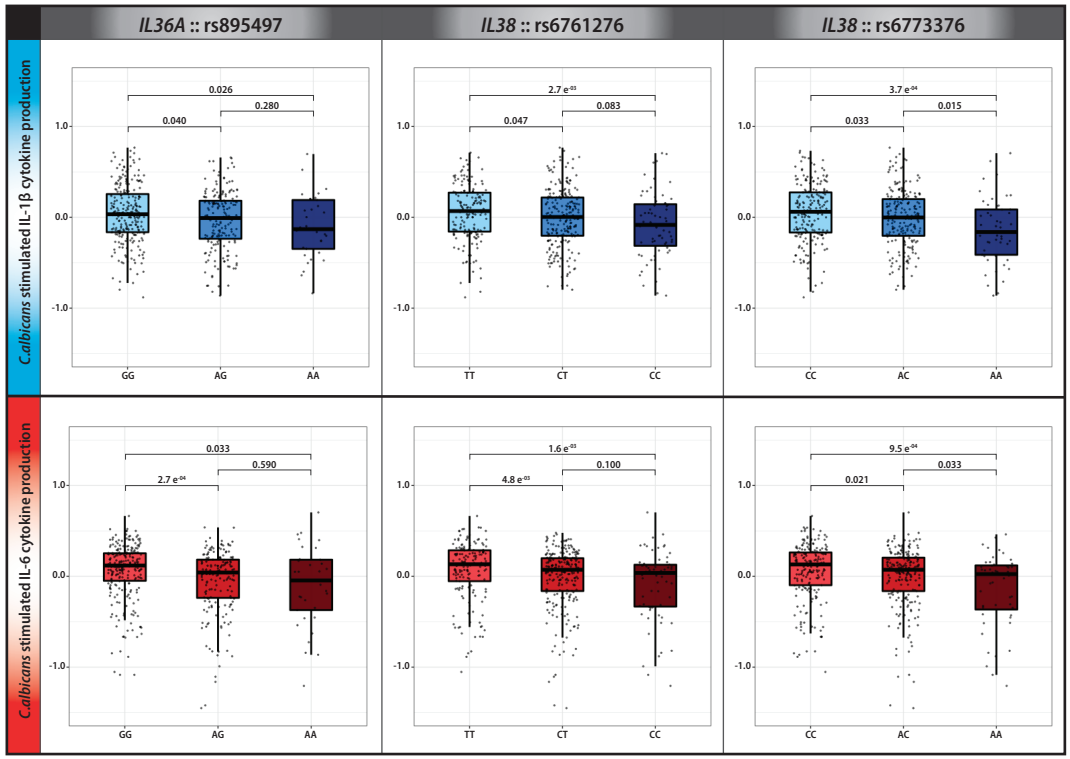
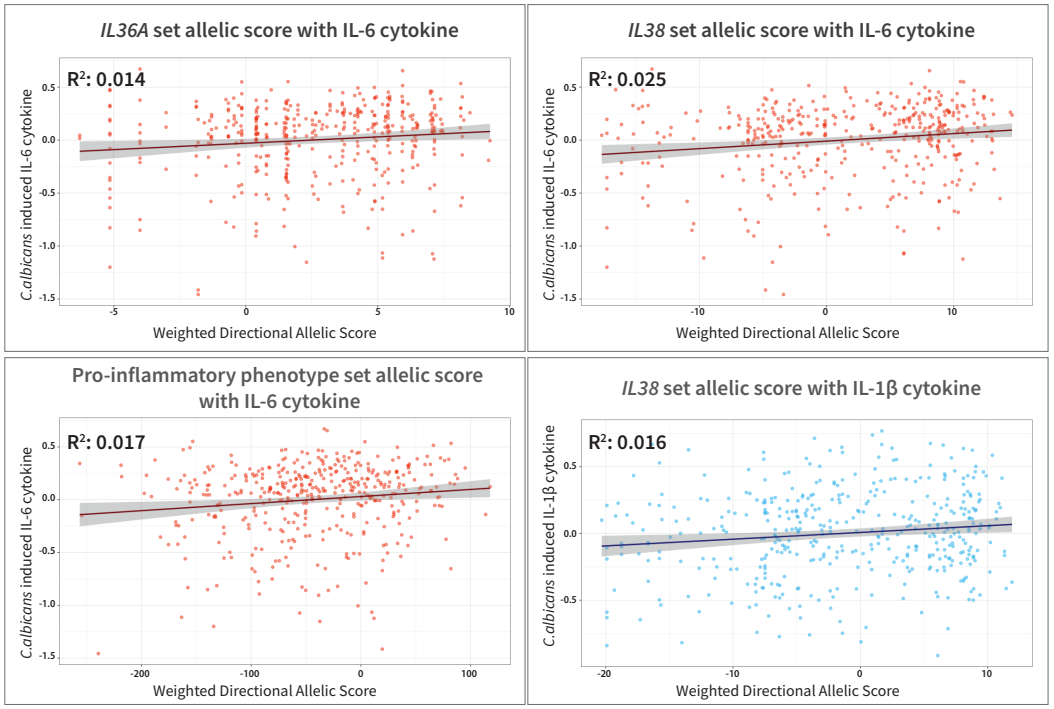


Figure 5. Coding and non-coding common variant sets are associated with *C. albicans* induced cytokine production. (A) shows that the residual IL-1β (top-panel in blue) and IL-6 (lower-panel in red) cytokine production for coding SNPs in *IL36A* and *IL38* decreases over the genotype categories. For all plots the ancestral allele is the minor allele and thus the genotype categories are ordered from left to right: homozygous alternative (IL-1β in light-blue and IL-6 in light-red), heterozygous (IL-1β in mid-blue and IL-6 in mid-red), homozygous ancestral (IL-1β in light-blue and IL-6 in light-red). (B) confirms that next to coding common variants, also non-coding common variants in *IL36A*, *IL38* and Pro-inflammatory phenotype sets are associated with *C. albicans* induced residual IL-6 cytokine production, and IL38 set with *C. albicans* induced residual IL-1β cytokine production. (C) shows the Beta-weighted, directional, allelic score in correlation with *C. albicans* induced residual IL-1β or IL-6 cytokine production. The straight line represents the linear model equation using method 'lm' with standard error of 0.95, and the R^2 is displayed in the plot.

Annotation: * = P-value < 0.05; ** = P-value < 0.01.

B. Non-coding and coding common variant set SKAT P-values

Set	all SNPs (n)	tag SNPs (n)	IL-1β SKAT P-value	IL-6 SKAT P-value
IL36A	103	17	0.248	0.049*
IL38	207	41	0.046*	0.007**
IL-30s subpathway	886	283	0.337	0.427
Pro-inflammatory phenotype	4541	1436	*NA*	0.019*
Anti-inflammatory phenotype	2996	994	0.222	0.280

C. Allelic score of significant non-coding and coding common variant sets