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Resource

Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity

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SUMMARY

Gut microbial dysbioses are linked to aberrant immune responses, which are often accompanied by abnormal production of inflammatory cytokines. As part of the Human Functional Genomics Project (HFGP), we investigate how differences in composition and function of gut microbial communities may contribute to inter-individual variation in cytokine responses to microbial stimulations in healthy humans. We observe microbiome-cytokine interaction patterns that are stimulus specific, cytokine specific, and cytokine and stimulus specific. Validation of two predicted host-microbial interactions reveal that TNFα and IFNγ production are associated with specific microbial metabolic pathways: palmitoleic acid metabolism and tryptophan degradation to tryptophol. Besides providing a resource of predicted microbially derived mediators that influence immune phenotypes in response to common microorganisms, these data can help to define principles for understanding disease susceptibility. The three HFGP studies presented in this issue lay the groundwork for further studies aimed at understanding the interplay between microbial, genetic, and environmental factors in the regulation of the immune response in humans.

INTRODUCTION

The gut microbiome is a crucial factor for shaping and modulating immune system responses, with gut microbial dysbioses linked to several autoimmune and immune-mediated diseases (Gevers et al., 2014; Kosiewicz et al., 2011; Paun et al., 2016). In the case of rheumatoid arthritis, this dysbiosis can be partially resolved after treatment (Zhang et al., 2015). While responding to pathogenic organisms is a main function of the immune system, recognition and tolerance of commensal bacteria are equally important for host health (Kosiewicz et al., 2011). Commensal microbes calibrate innate and adaptive immune responses and impact the activation threshold for pathogenic stimulations, in large part by producing small molecules that mediate host-microbial interactions (Donia and Fischbach, 2015). Short-chain fatty acids (SCFAs) are a classic example of how bacteria-derived molecules contribute to gut immune homeostasis (Lee and Hase, 2014; Thorburn et al., 2014). SCFAs serve as energy sources for gut epithelial cells, modulate cytokine production, and induce expansion of regulatory T cells. Consistent with these functions, SCFAs were implicated in anti-inflammatory properties of a mix of 17 human-derived Clostridia strains (Atarashi et al., 2013; Smith et al., 2013). Gut microbial communities harboring Clostridia are additionally beneficial in that they regulate immune function and epithelial permeability to food antigens to protect against allergen sensitization (Stefka et al., 2014).

Commensal microbiota also modulate systemic immune responses (Lee and Hase, 2014). While the epithelial barrier ensures that microorganisms are largely confined to the gut, microbial metabolites can penetrate the epithelial barrier, allowing them to enter and accumulate in the host circulatory system where they are sensed by immune cells (Dorrestein et al., 2014). For example, dietary tryptophan is metabolized by different gut bacteria resulting in distinct derivatives, including indole propionic acid, which is absorbed through the intestinal epithelium and enters the bloodstream. The biological activity of indole propionic acid, as is the case for many other small molecules, is largely unknown (Wikoff et al., 2009).

Given the many examples of commensal microbiota modulating immune responses, we investigated the relationship
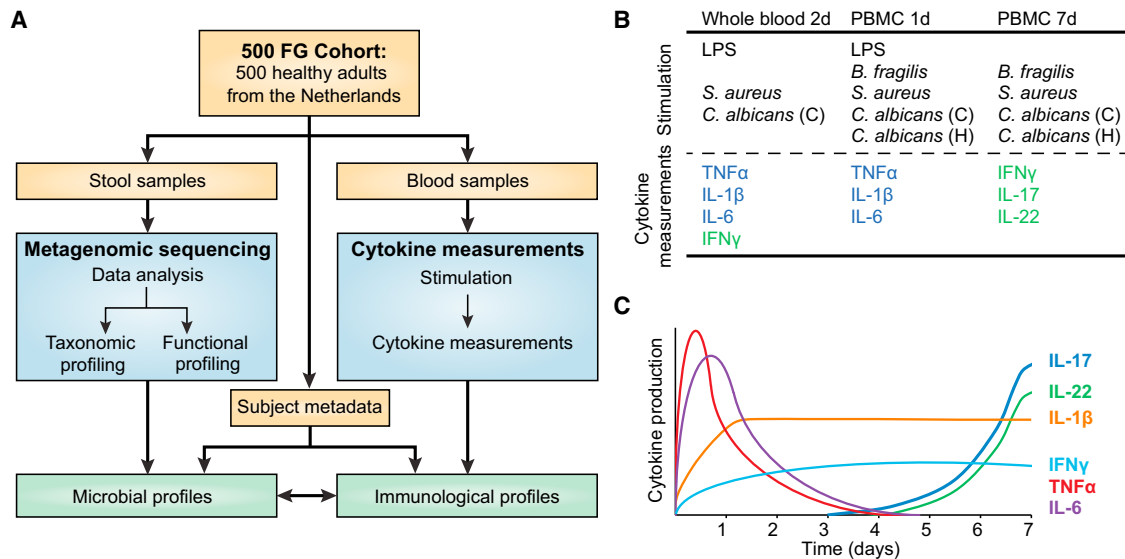
Figure 1. Linking Inter-Individual Variation in Immune Response to the Gut Microbiome in the 500FG Cohort
(A) The 500FG cohort comprises 500 healthy adults from the Netherlands. Stool samples were collected and subjected to metagenomic sequencing to infer gut microbial profiles. Concurrent blood samples were collected to measure the inflammatory cytokine response in connection with various microbial stimulations.
(B) Monocyte- (blue) (IL-6, TNFα, IL-1β) and lymphocyte-derived cytokines (green) (IFNγ, IL-17, IL-22) were measured in connection with three bacterial (LPS, *B. fragilis*, and *S. aureus*) and two fungal (*C. albicans* conidia and hyphae) stimulations in whole blood and/or PBMCs.
(C) Schematic illustrating production kinetics of different cytokines in PBMCs. IL-17 and IL-22 increase steadily during a 7-day period, whereas IL-6 and TNFα production is maximal in the first 24 hr and decreases thereafter. IFNγ reaches its maximum at 4–5 days, and IL-1β reaches its maximum level at 24 hr, after which a plateau is attained. Adapted from Ruschen et al. (1992) and van de Veerdonk et al. (2009).
See also Figure S1.

between inter-individual variation in gut microbial community composition and the inflammatory cytokine response to microbial stimulation in healthy individuals (Figure 1). To this end, we assessed potential functional relationships in multi-omic data including microbial and cytokine profiles from ~500 healthy individuals of Western-European genetic background in the 500 Functional Genomics (500FG) cohort from the Human Functional Genomics Project (HFGP) (Figure 1A; for exact sample numbers see Figures S1A and S1B). Several types of microbiome-cytokine interaction patterns were detected. We identified specific bacterial species and genera that are predicted to influence cytokine production capacity and found putative interactions between microbial metabolism and tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), a subset of which we experimentally validated. This work is complemented by two accompanying studies linking cytokine responses to host genetics (Li et al., 2016; [this issue of *Cell*]) and to host and environmental factors independent of the microbiome (ter Horst et al., 2016; [this issue of *Cell*]) yielding a comprehensive picture of the factors influencing human cytokine responses and host defense.

RESULTS

Study Overview

To identify associations between the gut microbiome and stimulus-induced cytokine responses, we collected stool samples from 500 healthy individuals to generate microbial taxonomic and functional profiles. Blood samples were taken concurrently from each individual and the cytokine response was measured ex vivo in peripheral blood mononuclear cells (PBMCs) and whole blood under five different microbial stimulations. Three stimulations were bacteria-derived (purified *Escherichia coli*-derived lipopolysaccharide [LPS] and *Bacteroides fragilis* representing Gram-negative bacteria, and *Staphylococcus aureus* representing Gram-positive bacteria) and two were fungal-derived (*Candida albicans* hyphae and conidia yeast) (Figures 1B and S1C). Although most stimulations were pathogens, we also included the common gut commensal organism *Bacteroides fragilis,* as this species can mediate development of the host immune system and was detected in 41% of all samples (Mazmanian et al., 2005). Three monocyte-derived cytokines (IL-1β, TNFα, IL-6) and three lymphocyte-derived cytokines (IFNγ, IL-17, IL-22) were measured at different times to capture their peak abundance (Figures 1B and 1C). We then used multivariate analyses to identify significant associations between microbial features and specific cytokine responses.

Between-Subject Variation Is Substantial Both in Cytokine Response to Stimulation and in Microbial Composition

We first analyzed the inter-individual variation in the inflammatory cytokine response and the gut microbiome composition, as this forms the basis for the identification of potential interactions between the two components (Figure 2A). The cytokine response to the stimulations was compared to the unstimulated state (RPMI) in whole blood and/or PBMCs (Figure 1B). While the
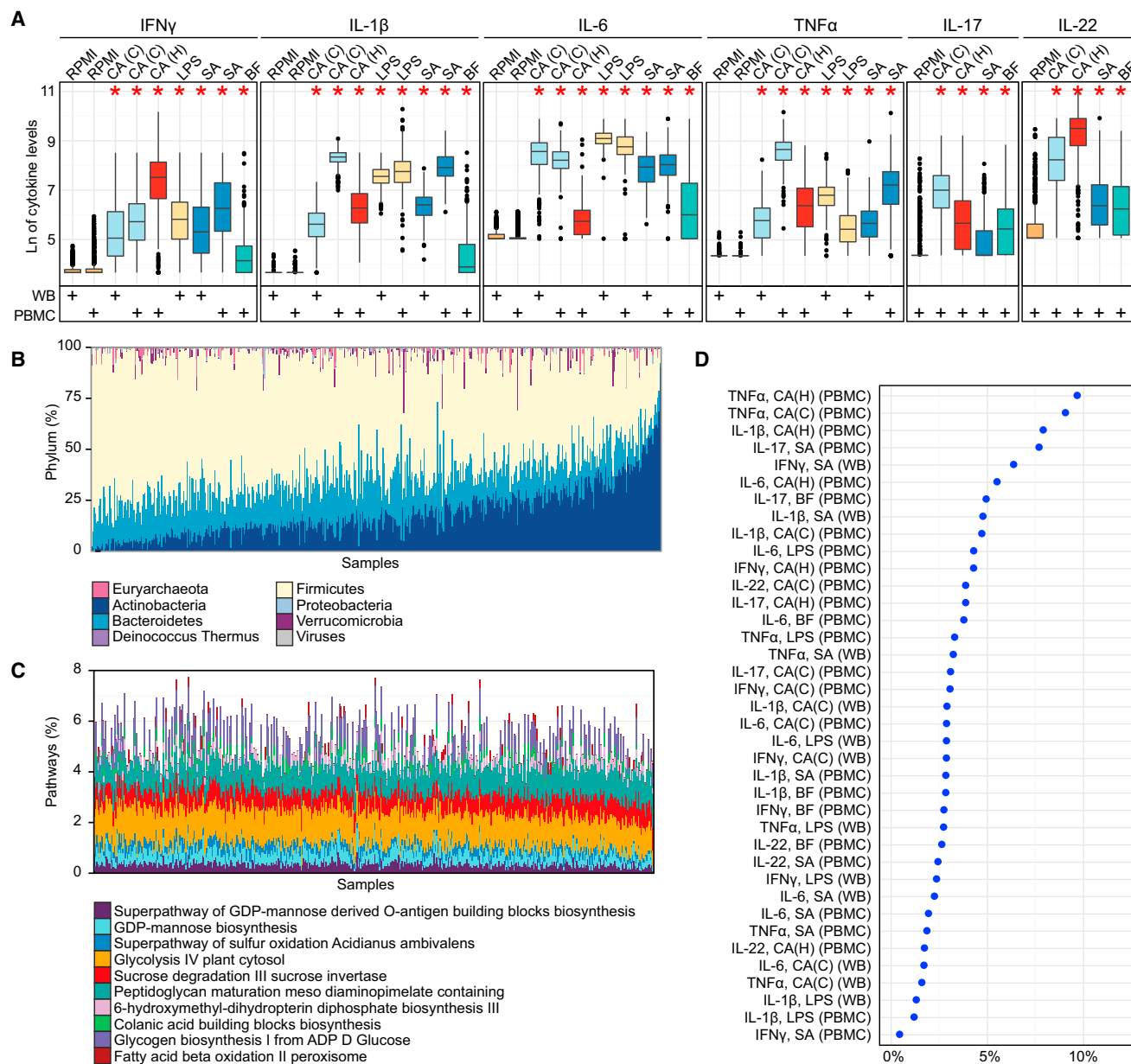
Figure 2. Healthy Individuals Show Significant Inter-Individual Variability in Stimulated Cytokine Responses and in Gut Microbiota

(A) Inter-individual variation in cytokine response. Each color represents a type of stimulation, also indicated on the upper x axis: CA(C), *C. albicans* conidia; CA(H), *C. albicans* hyphae; LPS, *E. coli*-derived lipopolysaccharide 100 ng; SA, *S. aureus*; BF, *B. fragilis*. Cell type is indicated below the x axis (WB, whole blood; PBMC, peripheral blood mononuclear cells). The y axis specifies the cytokine response. Each cytokine was measured in a non-stimulated control for each cell type (RPMI). Stars indicate significant differences in variation for each stimulatory response compared to controls, respectively for each cell type (Fligner-Killeen test, all $p < 1e-15$). (Note that *B. fragilis*-induced TNFα measurements were not considered for further analyses due to a small degree of variation across individuals.) Whole blood measurements are based on 456 individuals. TNFα, IL-1β, and IL-6 measurements in PBMCs (1 day) were available for 401 individuals; IFNγ, IL-17, and IL-22 measurements in PBMCs (7 days) are based on 462 individuals. Rectangles indicate the interquartile range (Q1–Q3).

(B) Taxonomic microbial profiles displaying phylum-level composition. Sample order was determined by the first principal component (PCA with Bray-Curtis distance).

(C) Functional microbial profiles displaying the abundance of the ten most variable MetaCyc pathways (based on variance). Samples are in the same order as in (B).

(D) The overall percentage of cytokine variation explained by species composition of the gut microbiome was 0.4%–9.7%. To avoid overestimation due to species-species correlations, we represented the microbiota through the first 20 principal coordinates (PCoA with Bray-Curtis distance) that explain ∼50% of the variance. The cytokine variance explained by these principal coordinates was estimated through permutation ANOVA by summing over the significant contributions ($p < 0.2$).

See also Figure S2.

unstimulated cells exhibited only a small degree of variation in cytokine levels, a high degree of inter-individual variation was observed in response to all stimulations. The difference in the degree of variation was significant for all stimulatory conditions compared to the unstimulated state for each cytokine and cell type, respectively (Fligner-Killeen test, all p < 1e-15). Generally, the intensity of the response to a particular stimulation was cytokine-dependent and in some cases also differed between whole blood and PBMCs. For most cytokines, the strongest response was induced by *C. albicans*; IL-6 was the only cytokine to show a stronger response to LPS stimulation.

In addition to immunological profiles, we used metagenomic sequencing reads to identify the species-level composition of the communities and their functional potential in the form of encoded genes and pathways. As expected, inter-individual variation in taxonomic composition was high, with even high-level phylum composition varying greatly across individuals (Figure 2B) (Human Microbiome Project Consortium, 2012). At the functional level, we detected many MetaCyc pathways with comparable levels across samples, with a subset of generally less abundant pathways varying more substantially between individuals (Figure 2C). The microbial profiles from the 500FG cohort are similar to those of LifeLines-DEEP, another healthy Dutch cohort within the HFGP (Fu et al., 2015; Tigchelaar et al., 2015; Zhernakova et al., 2016) and appear to represent a single population (Figure S1D). In contrast, when these data were compared with the Human Microbiome Project cohort (consisting of 242 healthy adults from the United States) (Human Microbiome Project Consortium, 2012), the two Dutch cohorts had higher levels of *Actinobacteria* and lower levels of *Bacteroidetes*. However, a high degree of inter-individual variability was observed within all cohorts.

Cytokine Variation Explained by the Microbiome

For each of the cytokine measurements, we quantified how much of the overall variation could be attributed to the gut microbiota (Figure 2D). To avoid an overestimation of variability due to species-species correlations, we used the first 20 principal coordinates to represent the species composition, accounting for ~50% of the variability in the microbial composition of the samples; thus, the variation we observed is a conservative estimate. The largest percentage of variation in the cytokine response was explained for TNFα in connection with *C. albicans* hyphae (9.7%) and conidia (9.0%) stimulation in PBMCs. For other stimulations, the microbiome accounted for <5% of the variation in the TNFα response, highlighting the stimulus-specific effect of the predicted microbiome-cytokine interactions. In addition, we assessed variation due to functional features using MetaCyc metabolic pathways and gene ontology (GO) categories. A maximum of 7.7% and 8.6% of the cytokine variation was explained by metabolic pathways and GO categories, respectively (Figure S2).

Three Classes of Microbiome-Cytokine Interaction Patterns

Because we observed significant inter-individual variation in cytokine responses and gut microbial profiles, we next assessed whether these factors were correlated. We predicted three types

of interaction patterns (IP), referred to as IP1 through IP3 (Figure 3A). The first interaction pattern (IP1) was stimulus-specific. In this case, gut microbial features were associated with the response of several cytokines, but only in connection with a particular type of stimulation. The second interaction pattern (IP2) describes cytokine-specific associations that were independent of the type of stimulation. In other words, a high/low response of a particular cytokine was connected to the abundance of a specific gut microbial species or function and observed in connection with several of the tested stimulations. Lastly, IP3 microbiome-cytokine interaction patterns were cytokine- and stimulus-specific. Here, a gut microbial component affected only the response of a particular cytokine and only in connection with a specific stimulation.

Gut Microbial Species and Genera Are Significantly Associated with Inflammatory Cytokine Responses

We performed pairwise correlation tests between microbial taxonomic composition at the species and genus levels with each cytokine in connection with a specific stimulation (Spearman correlation with Benjamini-Hochberg FDR correction $\alpha \leq 0.2$, Figures 3B and 3C). A total of 41 species and 20 genera were significantly associated with at least one potential interaction and associations were identified for all of the tested cytokines. All three interaction patterns were observed (Figure 3A) with the majority of associations being cytokine- and stimulus-specific (IP3). Three stimulus-specific (IP1) associations were observed, with *C. albicans* hyphae stimulation in connection with *Bacteroides xylanisolvens*, *Parabacteroides distasonis*, and *Coprococcus comes.* Furthermore, three potential cytokine-specific interactions (IP2) were identified. *Odoribacter splanchnicus* (Figure 3B) and the genus *Bilophila* (Figure 3C) were negatively correlated with TNFα production for LPS and *C. albicans* stimulations. *Barnesiella* was negatively associated with LPS and *B. fragilis*-induced IFNγ production (Figure 3C). Below, we detail the associations encountered for each of the stimulation types.

Taxonomic Associations with Fungal-Induced Cytokine Responses

Taxonomic features associated with fungal-induced cytokine responses were often connected to an altered IFNγ response (Figures 3B and 3C). This included common gut commensals such as *Dorea longicatena* and *Dorea formicigenerans*, where higher species abundances were connected to higher IFNγ levels in response to *C. albicans* hyphae. Both *Dorea* species can metabolize sialic acids, which are commonly found at terminal ends of mucins; release of these acids is implicated in mucin degradation, potentially increasing gut permeability (Crost et al., 2013). In contrast, another unclassified *Dorea* species was negatively correlated with the IFNγ response. Thus, different species of the same genus may affect the IFNγ response in opposite ways. We also observed different species from the same genus affecting different cytokines. For example, *Streptococcus parasanguinis* and *Streptococcus australis* were associated with IFNγ production, but other species such as the *Streptococcus mitis/oralis/pneumoniae* group were associated with IL-1β. Examples of microbial organisms predicted to affect multiple cytokines (IP1) include *Bifidobacterium*, which has been previously
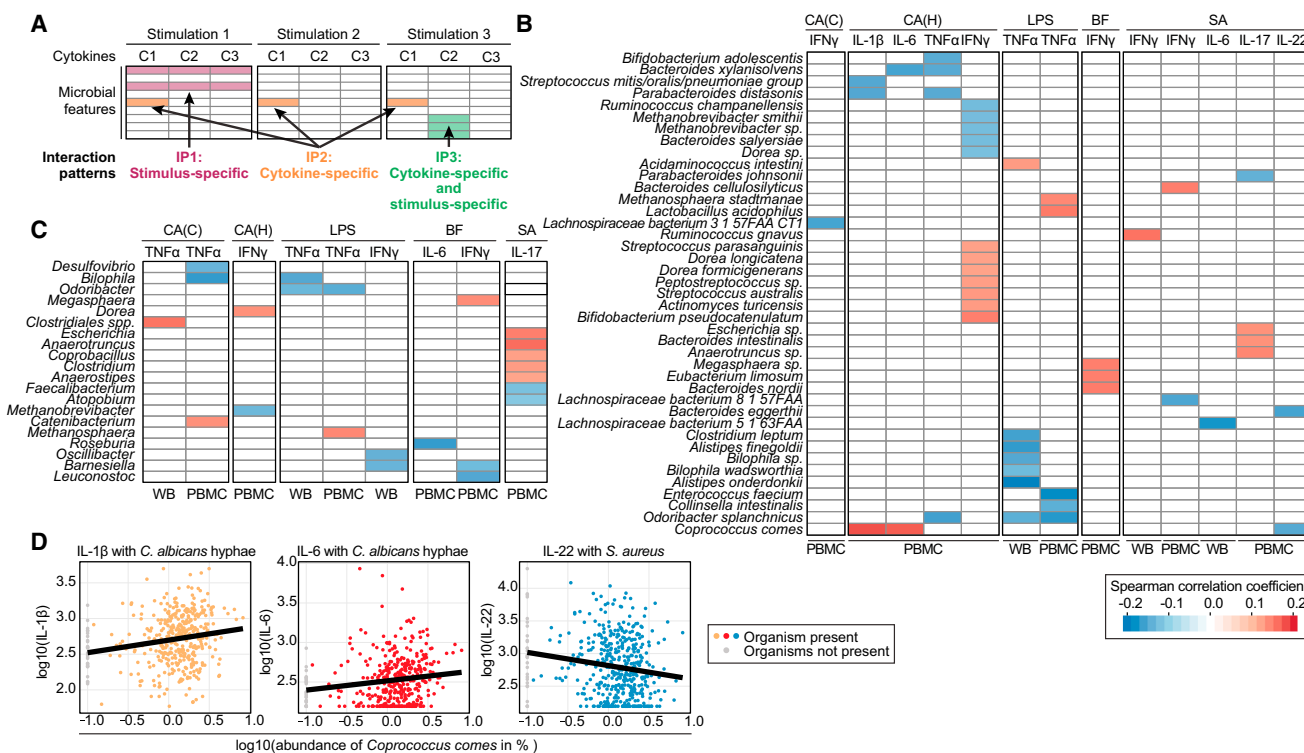
Figure 3. Significant Correlations between Gut Microbial Abundances and Cytokine Responses to Stimulations

(A) Three microbiome-cytokine interaction patterns were observed. Each colored cell represents a significant interaction between a specific microbial feature (y axis) and a stimulus-induced cytokine response (x axis). Interaction pattern 1 (IP1) refers to stimulus-specific interactions, where the same microbial feature is associated with several cytokines in connection with the same stimulation. IP2 describes cytokine-specific interactions, regardless of stimulation. IP3 refers to interactions that are cytokine- as well as stimulation-specific.

(B and C) Summary of species (B) and genus (C) associations with cytokine responses using Spearman correlation with Benjamini-Hochberg FDR correction ($\alpha \leq 0.2$). All species/genera were required to be detected in $\geq 3\%$ of all samples (corresponding to ≥ 14 samples). Only species/genera significantly associated with at least one cytokine response are displayed. CA(C), *C. albicans* conidia; CA(H), *C. albicans* hyphae; LPS, lipopolysaccharide 100 ng; BF, *B. fragilis*; SA, *S. aureus*; WB, whole blood; PBMC, peripheral blood mononuclear cells.

(D) A stimulus-specific association of *Coprococcus comes* was observed for IL-1β and IL-6 in connection with *C. albicans* hyphae stimulation. *C. comes* was also negatively correlated with *S. aureus*-induced IL-22 production. All displayed cell types are PBMCs.

See also Figure S3 and Tables S1, S2, S3, and S4.

associated with IFNγ production (López et al., 2011; Ménard et al., 2008). Here, we observed a positive correlation between *Bifidobacterium pseudocatenulatum* and IFNγ. In contrast, *Bifidobacterium adolescentis* was inversely correlated with TNFα production, further emphasizing the potential cytokine- and species-specificity of these associations. Further, *P. distasonis* was negatively associated with TNFα and IL-1β in response to *C. albicans* hyphae stimulation (Figure 3B). In human biopsies, *P. distasonis* was significantly more abundant in uninflamed versus inflamed tissue (Zitomersky et al., 2013). Consistent with this observation, oral administration of *P. distasonis* antigens attenuates intestinal inflammation during colitis in mice (Kverka et al., 2011).

These associations can also be used to form hypotheses regarding principles of host-microbial interactions. For instance, *C. comes* exhibited stimulus-specific associations with IL-1β and IL-6 in connection with *C. albicans* hyphae stimulation, suggesting that *C. comes* potentially modulates acute phase responses (Figure 3D). The acute phase response is a non-specific defense mechanism against microorganisms, characterized by a cascade involving stimulation of IL-1β and subsequent IL-6 production followed by fever and synthesis of acute-phase proteins. Both cytokines were positively correlated with *C. comes*, suggesting immunostimulatory properties of this microorganism on the acute phase pathway. In an accompanying manuscript examining host factors that influence cytokine response, ter Horst et al. (2016) found correlations between IL-1β and the acute phase protein alpha-1-antitrypsin (AAT). We therefore assessed the impact of *C. comes* on the circulating concentrations of AAT and found a positive correlation (r = 0.08, p = 0.08) (Figure S3) providing further evidence for the connection between *C. comes* and acute phase responses. Furthermore, *C. comes* demonstrated immunosuppressive properties in connection with IL-22 production induced by *S. aureus* (Figure 3D). The negative correlation of *C. comes* with IL-22 (in contrast to the positive correlations observed for IL-1β and IL-6) might at first appear contradictory, as IL-1β and IL-6 are both required for Th17 and IL-17/IL-22 production. However, complementary

data from the 500FG and 200FG projects show an absence of correlations between monocyte-derived cytokines (e.g., IL-1β and IL-6) and T helper cell responses (e.g., IL-22) (Li et al., 2016).

Taxonomic Associations with Bacterial-Induced Cytokine Responses

Three bacterial stimulations were used: LPS, *S. aureus*, and *B. fragilis*. For TNFα, IL-17, and IL-22, associations were only observed in connection with a specific bacterial stimulus (Figures 3B and 3C). A differential IFNγ response was observed in connection with *B. fragilis* and *S. aureus* at the species level (Figure 3B) and LPS and *B. fragilis* at the genus level (Figure 3C). In contrast, microbial associations with TNFα were exclusively detected in connection with LPS stimulation, suggesting that the gut microbiome influences TNFα production in a stimulus-dependent manner (Figures 3B and 3C; Tables S1, S2, S3, and S4). In total, three positive and nine negative associations were found at the species level for LPS-induced TNFα responses in whole blood and PBMCs. For example, negative associations between the microbiota and LPS-induced TNFα response involved multiple diet-sensitive bacteria, including *Alistipes spp.*, *Clostridium spp.*, and *Bilophila spp.* (in particular *B. wadsworthia*) (David et al., 2014; Devkota et al., 2012). This effect is particularly interesting given that anti-TNFα therapy is a common treatment for chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis, and these associations motivate potential microbiome-based therapeutic approaches.

S. aureus was the only bacterial stimulus where a differential Th17 response was associated with the microbiota (IL-17 and IL-22; Figures 3B and 3C). While the IL-17 and IL-22 response to *S. aureus* was generally lower compared to fungal-induced responses, a significant amount of variation was observed for all stimulations (see Figure 2A). For IL-17, five positive genus associations were identified, including *Clostridium*. This genus includes many species from *Clostridium* clades IV and XIV, which enhance T regulatory cell abundance and induce anti-inflammatory molecules, including IL-17 (Atarashi et al., 2013). Two negative genus associations with IL-17 production involved *Faecalibacterium* and *Atopobium* (Figure 3C). A prominent member of the *Faecalibacterium* genus, *F. prausnitzii*, inhibits IL-17 production in rats (Zhang et al., 2014). Our finding suggests that this may also be true in humans.

In contrast to these stimulus-specific examples, a differential IFNγ response was observed for all bacterial stimulations (Figures 3B and 3C). For LPS, for example, two negative associations were detected with *Oscillibacter* and *Barnesiella*. *Barnesiella* was additionally implicated in the *B. fragilis*-induced IFNγ response, representing a cytokine-specific interaction (IP2). One of the species-level associations involving *B. fragilis* stimulation was another *Bacteroides* species, *B. nordii*. However, in general the associations observed in connection with *B. fragilis*, a commensal gut bacterium, were not unique compared to the pathogenic stimulations. For example, the *S. aureus*-induced IFNγ response had three microbial associations, one being a positive association with *Bacteroides cellulosilyticus.* This observation suggests that while the various stimulations yield different associations, there does not appear to be a unique preferential association of *B. fragilis* with other *Bacteroides.*

Microbial Metabolites Attenuate Pathogen-Induced TNFα and IFNγ Responses

The immune system recognizes and reacts to small molecules produced by gut microbes, such as the previously mentioned example of SCFAs. These microbial functional pathways are often driven by multiple microorganisms, and hence, functional associations would not necessarily be detected in taxonomic pairwise association analyses. Therefore, we investigated correlations between cytokine production and MetaCyc metabolic pathways as well as GO categories, including biological processes and molecular functions, to identify functional categories implicated in differential cytokine responses (Figures 4A and 4B; Tables S5, S6, S7, and S8).

Functional Associations with Fungal-Induced Cytokine Responses

Analogous to the taxonomic analysis, we first focused on functional associations in connection with fungal-induced cytokine responses. The majority of functional associations were detected for the *C. albicans* conidia-induced TNFα and IFNγ response (Figures 4A and 4B; Tables S5, S6, S7, and S8). For TNFα, this observation was in contrast to the taxonomic analysis, where all associations were detected in connection with LPS stimulation (Figures 3B and 3C). Stimulus-specific interactions patterns involving TNFα and IFNγ are not surprising, as both are regulated by similar transcription factors in T cells, and IFNγ can further induce TNFα expression in dendritic cells.

Furthermore, 10 microbial functional features were predicted to affect the *C. albicans* conidia-induced production of TNFα and IL-22 in the same manner (Figure 4B). Both cytokines are produced by Th22 cells and can synergistically induce an effective innate immune response of epithelial cells during *C. albicans* infection, conferring protection in humans (Eyerich et al., 2011). Our results indicate that there are potential microbial factors that correlate with the production of both cytokines. In comparison to conidia, very few associations were detected for *C. albicans* hyphae, suggesting that the developmental state of the fungal pathogen is an important factor in determining host-microbial interactions.

Functional Associations with Bacterial-Induced Cytokine Responses

Microbial associations with the IL-6 response were detected for all bacterial stimulations (Figures 4A and 4B). For example, three MetaCyc pathways (Figure 4A) associated with the LPS-induced IL-6 response were involved in arginine degradation: cycloserine biosynthesis (PWY-7274), L-arginine and L-ornithine degradation (ORNARGDEG-PWY), and L-arginine, putrescine, and 4-aminobutanoate degradation (ARGDEG-PWY) (Table S5). L-arginine depletion mediated by myeloid cell arginase (ARG) has emerged as a fundamental mechanism of inflammation-associated immunosuppression (Bronte and Zanovello, 2005). Our data suggest that the microbiome may play an important role in arginine depletion and may need to be taken into consideration to address the immunomodulatory role of ARG. We also
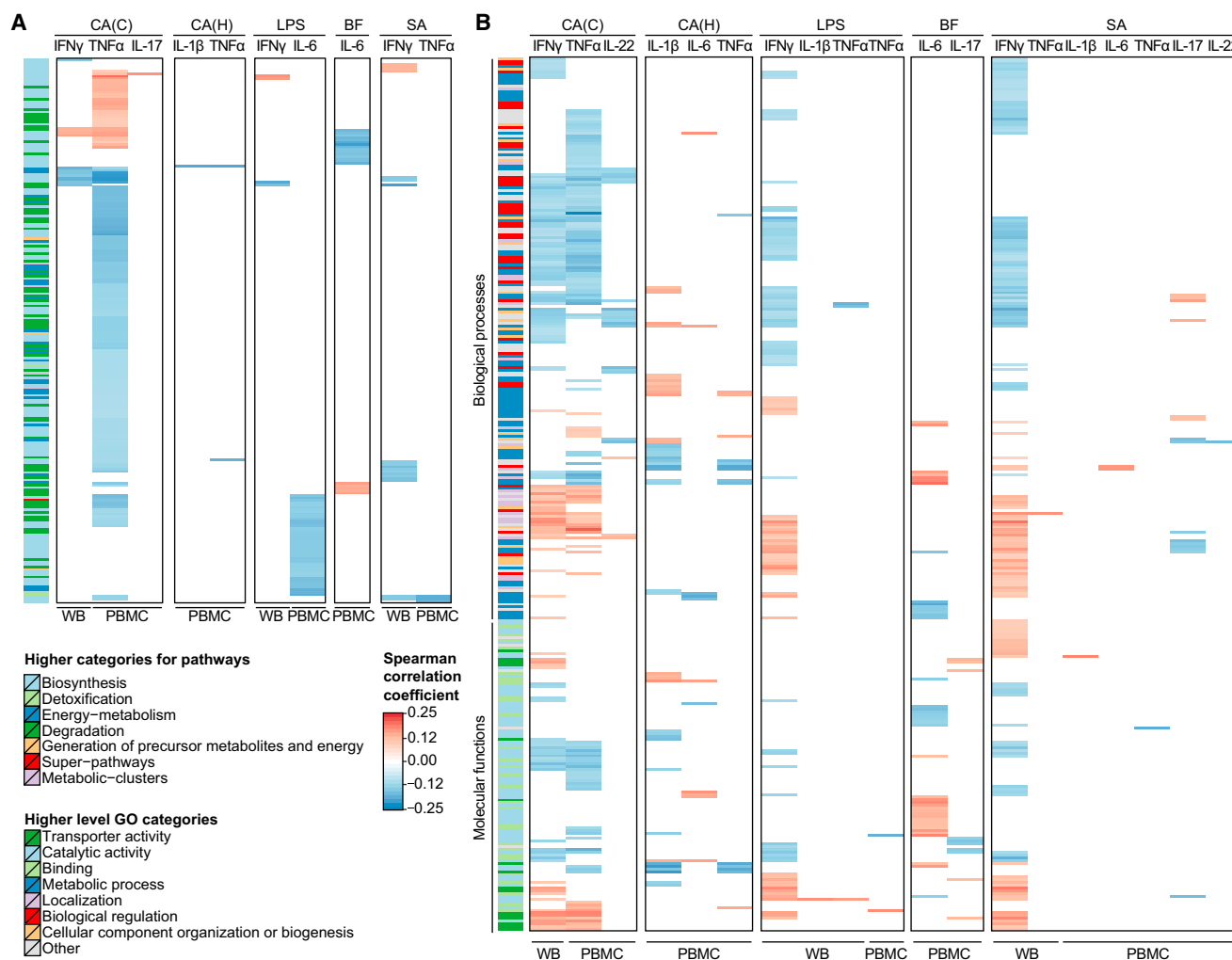
Figure 4. Significant Correlations between Functional Potential of the Gut Microbiome and Cytokine Responses

Functional summary for MetaCyc pathways (A) and Gene Ontology (GO) categories (B) using Spearman correlation with Benjamini-Hochberg FDR correction ($\alpha \leq 0.2$). All functional categories were required to occur in $\geq 3\%$ of the samples (≥ 14 samples). CA(C), *C. albicans* conidia; CA(H), *C. albicans* hyphae; LPS, lipopolysaccharide 100 ng; BF, *B. fragilis*; SA, *S. aureus*; WB, whole blood; PBMC, peripheral blood mononuclear cells. For MetaCyc pathways and GO categories, respectively, higher level functional categories are indicated on the left side.

See also Tables S5, S6, S7, and S8.

found a connection to the fungal-induced TNFα response, which was negatively correlated with two of these pathways (ORNARGDEG-PWY and ARGDEG-PWY).

Immunomodulatory Effects of Tryptophan and Tryptophol

Most functional associations in connection with bacterial stimulations were detected for IFNγ, involving a large number of cytokine-specific interactions (IP2) (Figure 4B). For example, microbial tryptophan degradation to tryptophol (MetaCyc pathway 5081) was negatively associated with IFNγ production in whole blood for *S. aureus*, LPS, and *C. albicans* conidia (Figure 5A). Tryptophan is metabolized in the gut into several molecules that play an important role in immune regulation, such as limiting inflammation by interacting with the aryl hydrocarbon receptor

(Lamas et al., 2016; Rothhammer et al., 2016), and both tryptophan depletion and downstream tryptophan metabolites can confer protection against excessive inflammation by immune inhibition (Opitz et al., 2007). To determine whether this observed decrease in IFNγ levels was due to a decrease in tryptophan or an increase in the resulting metabolite tryptophol, we experimentally validated these findings using a whole blood stimulation assay in healthy human donors. When tryptophan was added to whole blood, there was no effect on IFNγ production induced by LPS, *S. aureus*, or *C. albicans* conidia (Figure 5B). In contrast, tryptophol significantly decreased *C. albicans*- and LPS-induced production of IFNγ. Stimulation with *S. aureus* also showed a trend toward decreased IFNγ production when tryptophol was added (Figure 5B). These results show that microbially produced tryptophol has a strong effect on IFNγ production.
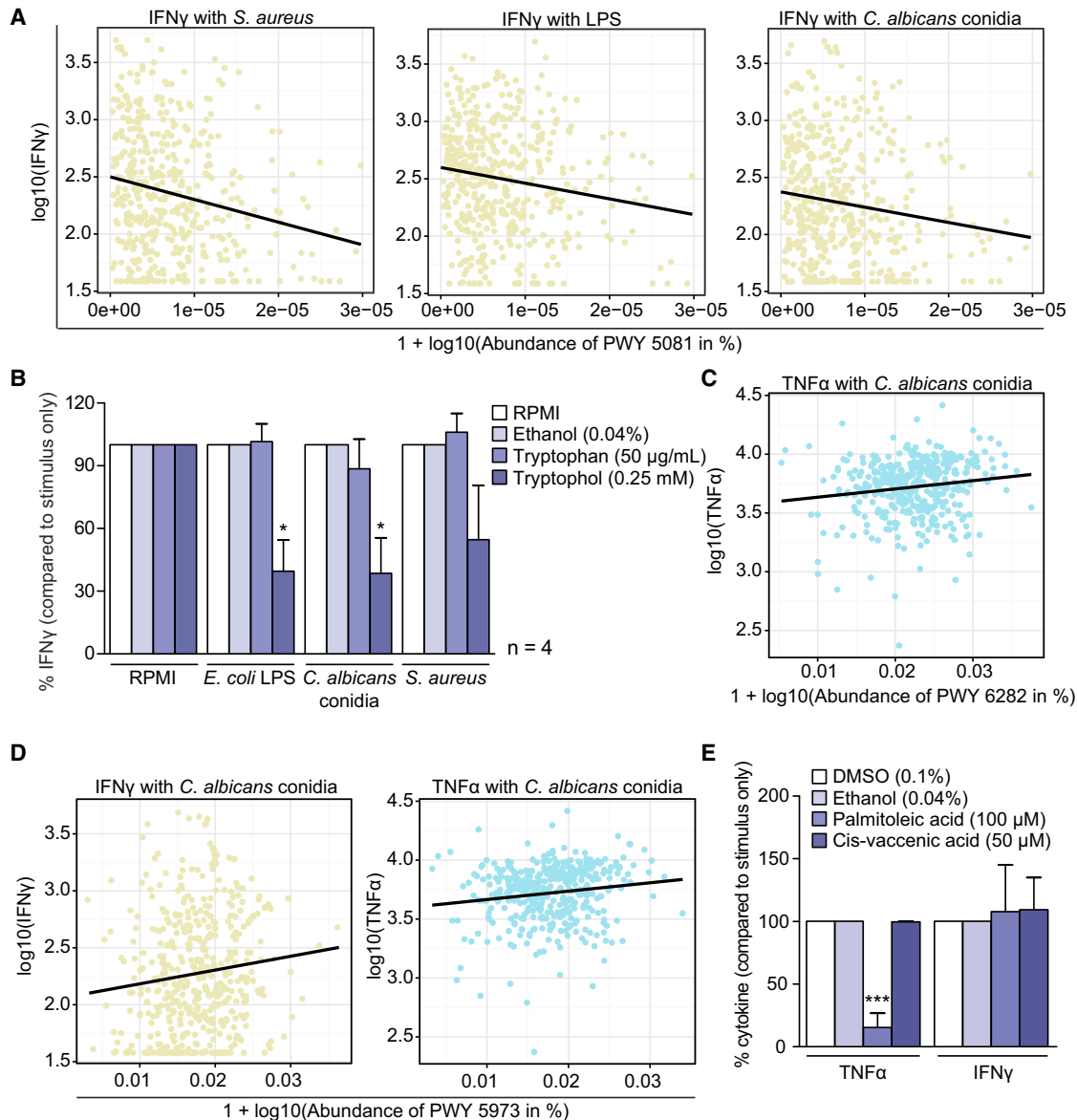
Figure 5. Immunomodulatory Effects of Tryptophol and Palmitoleic Acid

(A) L-tryptophan degradation to tryptophol (MetaCyc PWY 5081) was negatively associated with IFNγ in connection with *S. aureus*, LPS, and *C. albicans* conidia in whole blood.

(B) Whole blood was stimulated with LPS, *C. albicans* conidia, or *S. aureus* in the presence or absence of tryptophan (50 μg/mL) or tryptophol (0.25 mM) and IFNγ concentrations were measured in supernatants for 4 donors. Group differences are expressed as percentage change compared to RPMI (tryptophan) or ethanol (tryptophol) and analyzed by paired *t* test. *p < 0.05. Bars indicate the mean percentage change with SEM. Mean values for RPMI controls in pg/mL: RPMI, 8; LPS, 370; *C. albicans* conidia, 115; *S. aureus*, 405. Mean values for ethanol controls in pg/mL: RPMI, 8; LPS, 219; *C. albicans* conidia, 131; *S. aureus*, 91.

(C) Palmitoleic acid biosynthesis (MetaCyc PWY 6282) in PBMCs was positively correlated with *C. albicans* conidia-induced TNFα production.

(D) *Cis*-vaccenate biosynthesis (MetaCyc PWY 5973) was positively correlated with IFNγ (in whole blood) and TNFα (in PBMCs) in connection with *C. albicans* conidia stimulation.

(E) PBMCs from five to six healthy volunteers were stimulated with *C. albicans* conidia in the presence or absence of palmitoleic acid and *cis*-vaccenate (*cis*-vaccenic acid). Cytokine concentrations were measured in supernatants. Group differences are expressed as percentage change compared to ethanol or DMSO measurements and analyzed by paired *t* test. ***p < 0.001. Bars indicate the mean percentage change with SEM. Mean values in pg/mL: TNFα (DMSO, 10,000; ethanol, 10,000; palmitoleic acid, 1,504; *cis*-vaccenate, 9,957); IFNγ (DMSO, 1,370; ethanol, 1,571; palmitoleic acid, 976; *cis*-vaccenate, 2,241).
See also Figure S4.

Palmitoleic Acid Can Inhibit the Pro-inflammatory Cytokine Response

Two related MetaCyc pathways showed positive correlations with TNFα in response to *C. albicans* conidia stimulation: palmitoleic acid biosynthesis (MetaCyc PWY 6282) and *cis*-vaccenate biosynthesis (MetaCyc PWY 5973) (Figures 5C and 5D). *Cis*-vaccenate biosynthesis was also positively associated with IFNγ production. Palmitoleic acid biosynthesis has a branch point from a palmitoleoyl [acp] to the *cis*-vaccenate pathway (Figure S4A). Thus, increased activity in the palmitoleic acid biosynthesis pathway could result in either more palmitoleic acid or more *cis*-vaccenate if the *cis*-vaccenate pathway were also active. In the second scenario, an increase of *cis*-vaccenate would utilize the substrate for palmitoleic acid biosynthesis, resulting in less palmitoleic acid production. Given that our associations with *cis*-vaccenate biosynthesis have a positive correlation with TNFα and IFNγ, it is possible that increased palmitoleic acid corresponds to decreased cytokine production. We tested how the presence of these molecules affects *C. albicans* conidia-induced cytokine production in human PBMCs (Figure 5E). Palmitoleic acid decreased TNFα production but had no effect on IFNγ. However, *cis*-vaccenate (*cis*-vaccenic acid) did not influence cytokine production under any of the tested stimulations (Figure 5E). Interestingly, palmitoleic acid displayed an anti-inflammatory effect in connection with all of the monocyte-derived cytokines (IL-1β, TNFα, IL-6), but had no effect on lymphocyte-derived cytokines (IFNγ, IL-17, IL-22) (Figures S4B and S4C).

DISCUSSION

The HFGP is a comprehensive study that aims to identify the most important factors influencing cytokine production capacity in healthy humans. In three complementary studies, we report the environmental, genetic, and microbiome factors associated with cytokine production induced by different microbial stimulations in a cohort of 500 healthy volunteers. While accompanying studies demonstrate the impact of environmental and host factors (genetic and non-genetic) for the modulation of cytokine production (Li et al., 2016; ter Horst et al., 2016), in this study we provide evidence that the gut microbiome also has biologically relevant effects.

Our analysis indicates that inter-individual variation in cytokine response is linked to specific microbial organisms as well as microbial functions. The majority of detected associations were both cytokine- and stimulus-specific (IP3), suggesting that the immune system recognizes and interacts with microbial organisms and products with high specificity and that these microbial factors are associated with a particular immunological phenotype. In particular, a broad range of microbial functional features was associated with cytokine responses. This observation suggests that modulation of host defense by the microbiota may be exerted mainly through the release of intermediary common mediators (such as metabolites) rather than direct interaction between specific microorganisms and immune cells. This finding constitutes the first important conclusion that can be drawn from our data.
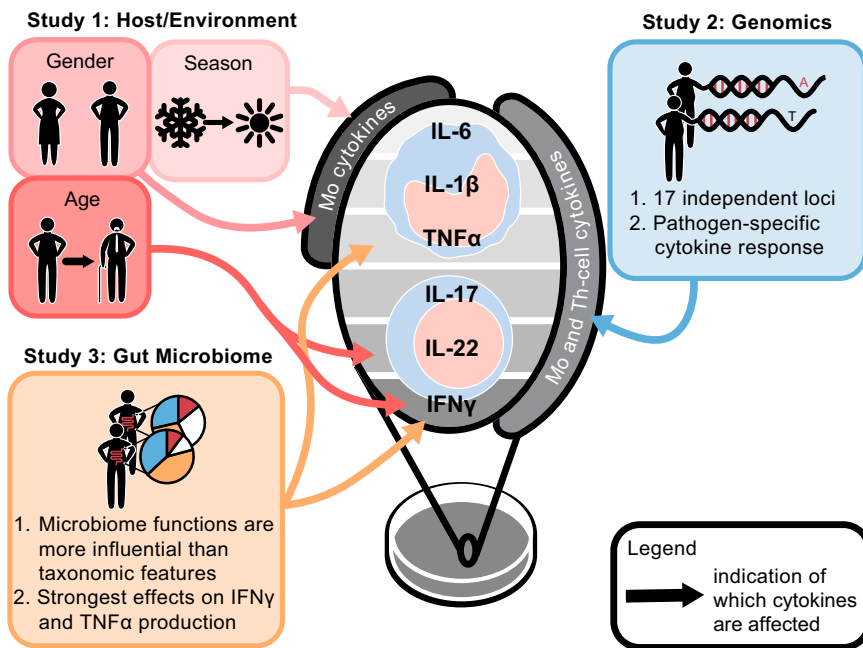
An important role for metabolites in microbiome-cytokine interactions is supported by the fact that a large proportion of the metabolites in the blood originate from the gut (Sridharan et al., 2014;

Wikoff et al., 2009), as well as our findings of the strong impact of microbial metabolic processes on cytokine production (Figure 4). Among these metabolic processes, we experimentally validated the important impact of microbial tryptophan metabolism, a pathway known to modulate cytokine production at the level of host metabolism (Moffett and Namboodiri, 2003; Nowak et al., 2012). Here, we demonstrate that microbial tryptophan metabolism strongly influences cytokine production. Interestingly, this effect is mainly mediated by the tryptophan metabolite tryptophol, which has strong inhibitory effects on the TNFα response. However, the inhibitory effect depends on the particular pathogenic stimulation. We also validated a predicted interaction involving palmitoleic acid metabolism. Palmitoleic acid inhibits apoptosis induced by a combination of IL-1β and IFNγ (Welters et al., 2004), but a specific effect on IFNγ synthesis has not been previously reported. Interestingly, we not only validated this relationship in human cell stimulation assays, but also demonstrated a specific effect of palmitoleic acid on monocyte-derived cytokines (TNFα, IL-1β, IL-6) but not lymphocyte-derived cytokines (IFNγ, IL-17, IL-22). The molecular mechanisms responsible for this specificity remain to be elucidated in future studies. These validations serve as proof-of-principle of how predicted associations can be used to provide insight into host-microbial interactions.

In addition to the observations concerning modulation of cytokine production capacity by microbial functions, we also predicted interactions of specific microbial species and genera, including the effect of *C. comes* on cytokines of the acute phase pathway (IL-1β and IL-6). The production of IL-1β and IL-6 is regulated largely at a genetic level (Li et al., 2016) and less influenced by microorganisms. The physiological relevance of this finding is supported by our subsequent analysis showing that *C. comes* is associated with circulating concentrations of the acute phase protein AAT. This connection was observed despite the fact that AAT was measured during health, not after microbial challenge or infection.

Insight into gut microbiota and immune system interactions is a crucial step in understanding immune-mediated and infectious diseases. For example, differential abundance of specific gut bacteria was associated with cytokine responses and may impact disease susceptibility. This effect was exerted directly on the intrinsic cytokine production capacity of the immune cells, rather than by influencing the number of cells in circulation: no effect of microbiota composition on the most important immune cell populations (T/B lymphocytes, monocytes, neutrophils, NK cells) was detected in the present study (data not shown).

Evaluating the results of the three studies presented in this issue, it appears that variation at many levels affects the inflammatory response. An earlier study showed that host genetic variants modulate pathogen sensing in dendritic cells stimulated with microbial ligands (Lee et al., 2014), and we now present a much broader assessment of the genetic (Li et al., 2016) and environmental factors (ter Horst et al., 2016) that modulate cytokine production. Non-genetic factors such as age, gender, and season have a strong influence on cytokine production capacity, and genome-wide analyses have identified 17 independent loci that influence cytokine production at a genome-wide level. Here, we demonstrate that the microbiome also plays a key role in the regulation of cytokine production. This conclusion is supported by studies showing that perturbations such as

Study 1: Host/Environment

Gender   Season

Age

Study 3: Gut Microbiome

1. Microbiome functions are more influential than taxonomic features
2. Strongest effects on IFNγ and TNFα production

Mo cytokines

IL-6

IL-1β

TNFα

IL-17

IL-22

IFNγ

Mo and Th-cell cytokines

Study 2: Genomics

1. 17 independent loci
2. Pathogen-specific cytokine response

Legend

indication of which cytokines are affected

Figure 6. Environmental, Host Genetic, and Gut Microbial Factors Impact Human Cytokine Responses

The impact of host environmental factors (ter Horst et al., 2016), host genetics (Li et al., 2016), and the gut microbiome (this study) on stimulus-induced cytokine responses was assessed in three complementary studies of the HFGP. While gender and seasonality were the main environmental factors affecting the response of monocyte-derived cytokines, age was associated with IL-22 and IFNγ production. Further, 17 independent loci were implicated in specific differential cytokine responses of monocyte- and Th-derived cytokines. Lastly, gut microbial functions were more influential on cytokine production than taxonomic features, where the strongest effects were observed for the stimulus-induced IFNγ and TNFα responses.

antibiotic treatment greatly impact development of autoimmune diseases such as psoriasis (Zanvit et al., 2015) and are associated with allergic asthma susceptibility (Russell et al., 2012). In addition, the use of antibiotics is associated with a more pronounced dysbiosis in children with Crohn's disease, implicating perturbations of the gut microbiota in disease (Gevers et al., 2014).

The HFGP provides the opportunity to evaluate the importance of these host-dependent factors. Although host genetic variability and microbiome composition both influence cytokine production, our analyses suggest a greater impact of host genetics, explaining 25%–50% of the variability of some cytokine responses, compared to the microbiome, explaining only up to 10% of cytokine variability.

Several limiting factors can introduce noise to the microbiome data, resulting in smaller correlation coefficients. First, any experimentally obtained data contain noise; for example, the immune cells were stimulated ex vivo, outside the environment where the cells were exposed to microbial factors, potentially weakening the detectable effects of host-microbial interactions. Second, simultaneously occurring host-microbial associations may affect each other adversely. Importantly, the effect size does not necessarily provide knowledge about biological significance, as illustrated by the experimental validation of two functional associations, providing conclusive evidence for the specificity of these host-microbial interactions.

The HFGP studies provide a comprehensive assessment of the effects of host and environmental factors on cytokine production capacity at a population level. Integrated approaches initiated in HFGP enabled us to identify important general interaction characteristics of different biological traits in the human hosts (Figure 6). First, general characteristics of the host such as age and gender have an important impact on inflammation. Men display a higher production of monocyte-derived proinflammatory cytokines, whereas women have a greater Th17 response (ter Horst et al., 2016). These differences may provide a partial explanation for differences in pathologies between men and women: men are more susceptible to metabolic and cardiovascular diseases, whereas women more often have autoimmune diseases (Regitz-Zagrosek, 2006; Regitz-Zagrosek et al., 2006). In addition, a defective IFNγ production capacity has been observed in elderly people (Ouyang et al., 2002), which may explain their increased susceptibility to infections. Second, genomic approaches in HFGP demonstrate the importance of host genetics for determining cytokine responses. Although this concept is not new, here we have analyzed a large healthy population in a genome-wide fashion and report 17 new genetic loci associated with specific responses (Li et al., 2016). Third, we found that the effects of both genetic and microbiome factors are mainly stimulus- and cytokine-specific, and interesting new patterns emerged: TNFα and IFNγ production capacity appear to be more strongly influenced by the microbiome, whereas other cytokines such as IL-1β, IL-6, and Th17-derived IL-17 and IL-22 exhibit fewer, but more specific, associations with the gut microbiota. This finding is in line with genetic data showing an opposite trend for monocyte- and Th-derived cytokines (Li et al., 2016). These findings may have important consequences for approaches to immunotherapy targeting specific cytokines to alter immune responses: while inhibition of cytokines that are strongly influenced by the microbiome can potentially be modulated through diet, elimination of specific species, or fecal microbiota transplantation, host genetics-modulated cytokines may be more effectively targeted through inhibitory pharmacological approaches (e.g., neutralizing monoclonal antibodies).

Through these three complementary HFGP studies assessing the impact of the host microbiome (this study), environmental (ter Horst et al., 2016), and host genetic factors (Li et al., 2016), we are able to glimpse the complexity of the mechanisms driving the variability of human cytokine responses. These studies open the possibility for future similar investigations in patient

groups to identify the variation and disturbances responsible for their diseases in a personalized fashion, thereby providing the basis for precision medicine.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.10.020.
An audio PaperClip is available at http://dx.doi.org/10.1016/j.cell.2016.10.020#mmc2.

AUTHOR CONTRIBUTIONS

M.S. performed the metagenomics data analyses and correlation analyses. S.P.S., M.J., M.O., A.K., and M.J.B. performed the immunological assays and processed and analyzed the data. M.S., H.V., E.A.F., C.H., M.G.N., and R.J.X. interpreted the results. M.S., E.A.F., and C.H. led the computational methods and research development. S.P.S., M.J., and M.O. collected clinical samples. S.P.S., T.J., and L.J. performed functional validation experiments. M.S., S.P.S., H.V., C.H., M.G.N., and R.J.X. assembled and wrote the paper. M.S., H.V., M.G.N., and R.J.X. served as project leaders. J.F., A.Z., L.A.B.J., C.W., M.G.N., and R.J.X. designed the cohort study. C.H., C.W., M.G.N., and R.J.X. served as principal investigators.

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