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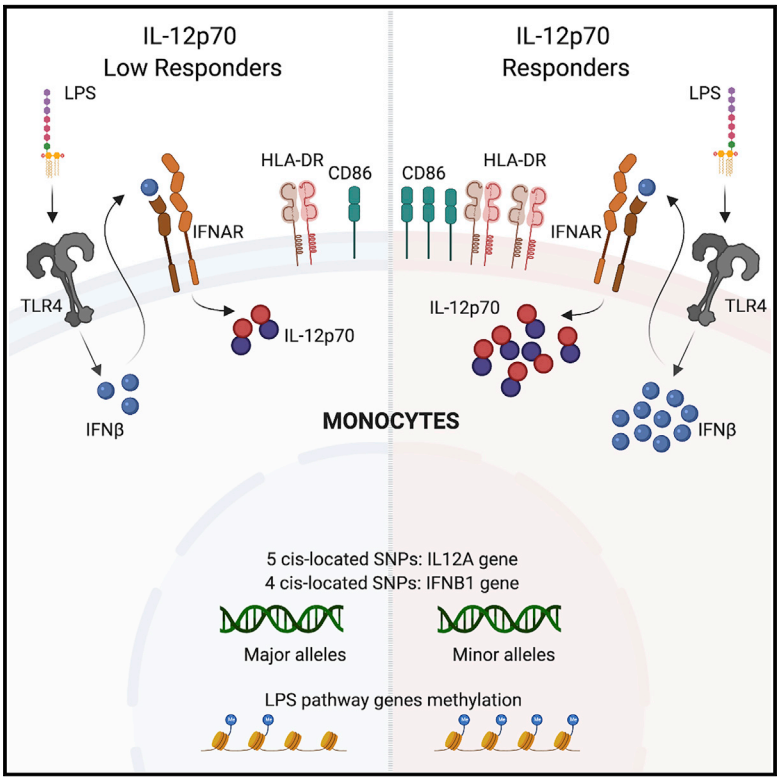
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Article

Cell Reports

Early IFNβ secretion determines variable downstream IL-12p70 responses upon TLR4 activation

Graphical abstract



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In brief

Posseme et al. show a quarter of individuals secrete low levels of IL-12p70 after bacterial infection. Using 1,000 healthy donors, they show IL-12p70 is dependent upon upstream IFNβ, different cellular populations, and genetic changes. Levels of IFNβ and IL-12p70 are also altered in acute SARS-CoV-2 and chronic HCV infection.

Highlights

- High variability is observed in IL-12p70 after bacterial stimulation of whole blood
- Upstream IFNβ secretion by monocytes is key to the regulation of LPS-induced IL-12p70
- Systems immunology reveals cellular, genetic, and epigenetic regulators of IL-12p70
- IFNβ/IL-12p70 pathway is perturbed in acute SARS-CoV-2 and chronic HCV infection

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Early IFNβ secretion determines variable downstream IL-12p70 responses upon TLR4 activation

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SUMMARY

The interleukin-12 (IL-12) family comprises the only heterodimeric cytokines mediating diverse functional effects. We previously reported a striking bimodal IL-12p70 response to lipopolysaccharide (LPS) stimulation in healthy donors. Herein, we demonstrate that interferon β (IFNβ) is a major upstream determinant of IL-12p70 production, which is also associated with numbers and activation of circulating monocytes. Integrative modeling of proteomic, genetic, epigenomic, and cellular data confirms IFNβ as key for LPS-induced IL-12p70 and allowed us to compare the relative effects of each of these parameters on variable cytokine responses. Clinical relevance of our findings is supported by reduced IFNβ-IL-12p70 responses in patients hospitalized with acute severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection or chronically infected with hepatitis C (HCV). Importantly, these responses are resolved after viral clearance. Our systems immunology approach defines a better understanding of IL-12p70 and IFNβ in healthy and infected persons, providing insights into how common genetic and epigenetic variation may impact immune responses to bacterial infection.

INTRODUCTION

Upon infection, a balanced immune response is crucial to respond to the pathogen while maintaining homeostasis. Insufficient immune responses can increase susceptibility to infections, while an overactive immune system may lead to autoimmune disorders, such as type 1 diabetes or lupus (Atkinson et al., 2014; Tsokos et al., 2016). Although cytokines are essential regulators of both homeostasis and inflammatory responses, few studies have been conducted in humans to understand the causes of variance in healthy individuals before applying this understanding to disease cohorts (Brodin et al., 2015; Enroth et al., 2014; Li et al., 2016b; Ter Horst et al., 2016). We hypothesized that understanding the underlying reasons of how variance exists will provide fundamental understanding in disease states, with potential clinical relevance.

A crucial cytokine for response to infection is interleukin-12p70 (IL-12p70), a heterodimeric pro-inflammatory cytokine composed of the p35 and p40 subunits, which induces Th1 responses (Kobayashi et al., 1989). IL-12p70 induces the proliferation and cytotoxicity of NK cells as well as the differentiation and proliferation of Th1 cells, primarily through the induction of interferon γ (IFNγ) and subsequent Jak/STAT signaling (Trinchieri, 2003). Although p35 requires binding to p40 to be secreted (Wolf et al., 1991), p40 can function autonomously as a monomer (IL-12p40) or homodimer (IL-12p80) or it can bind

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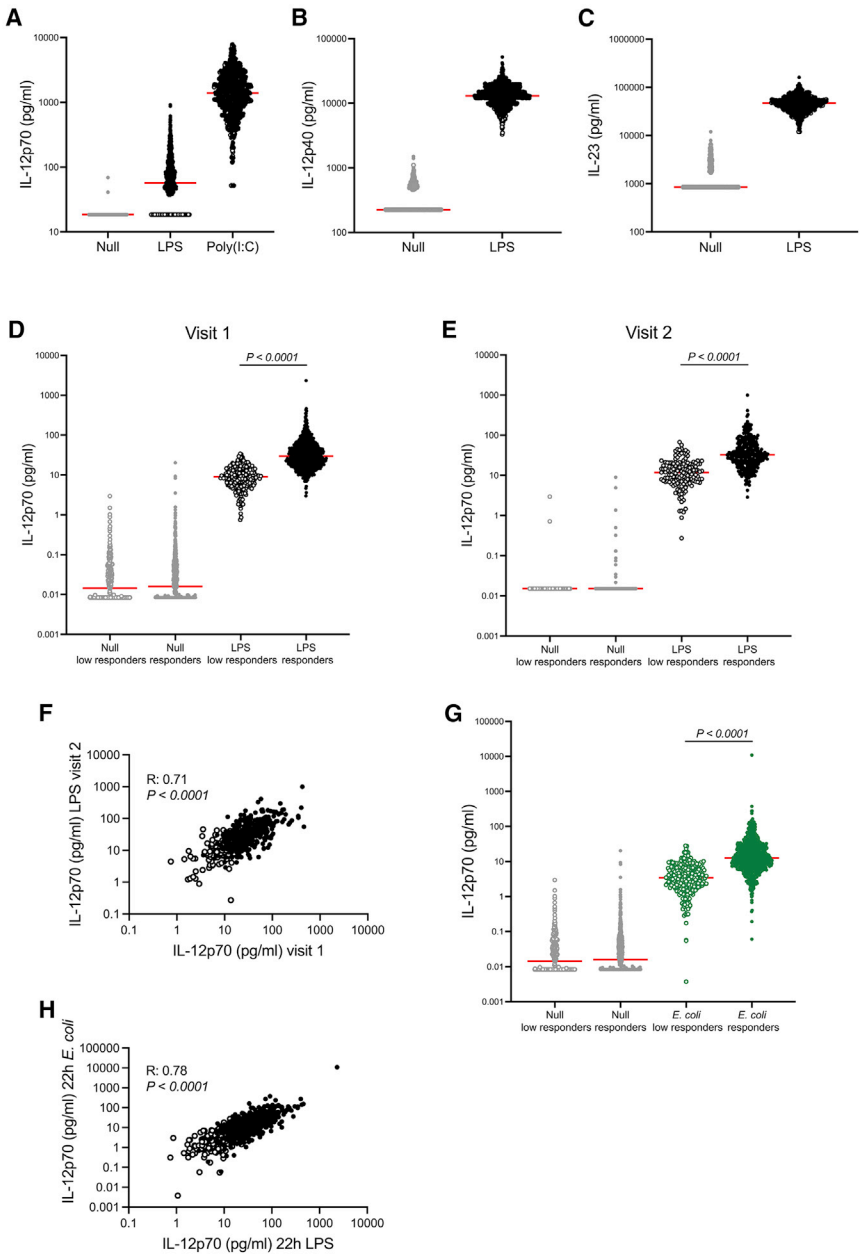


Figure 1. IL-12p70 variability in healthy donors

(A–E) IL-12p70 (A) measured by Luminex in healthy donors (n = 1,000) after stimulation of whole blood with null, LPS, or poly (I:C). IL-12p40 (B) and IL-23 (C) proteins measured by Luminex in healthy donors (n = 1,000) after null and LPS stimulation. IL-12p70 secretion measured by Simoa after null and LPS stimulation at two separate time points (visit 1 n = 1,000, D; visit 2 n = 500, E).

(F) Correlation of IL-12p70 secretion after LPS 22 h stimulation at two separate time points. Pearson correlation was performed on log10 values.

(G) IL-12p70 secretion measured by Simoa in the two groups of *Milieu Interieur* IL-12p70 responders after null and *E. coli* stimulation. Red lines indicate the median value for each group. p values were determined by an unpaired Student's t test using log10 values.

(H) Correlation of IL-12p70 secretion measured by Simoa after *E. coli* and LPS 22 h stimulation. Pearson correlation was performed using log10 values. Empty symbols represent IL-12p70 low responders as defined by LPS 22 h whole-blood stimulation (A); full symbols represent IL-12p70 responders.

to the p19 subunit to form the pro-inflammatory cytokine IL-23 (Ling et al., 1995; Oppmann et al., 2000). IL-12p40 can antagonize IL-12p70-driven responses by competing for the receptor, whereas IL-23 facilitates Th17 responses (Vignali and Kuchroo, 2012). Given this complexity, a better understanding of the factors that determine the variable functions of IL-12 cytokines may have broad implications for understanding diverse immune functions in homeostasis and disease.

We previously reported high levels of inter-individual variability in IL-12p70 responses to lipopolysaccharide (LPS) stimulation in a small cohort of healthy donors (Duffy et al., 2014). Here, we dissected the factors behind this variability in 1,000 healthy individuals of the *Milieu Interieur* (MI) cohort. Using standardized immunomonitoring tools, we identified that a quarter of healthy individuals consistently secreted low levels of IL-12p70 cytokine after LPS stimulation. We applied a systems biological approach integrating proteomic, transcriptomic, cellular, epigenetic, and genomic datasets to assess environmental and intrinsic factors driving IL-12p70 variability. From this, we identified upstream variability in IFNβ as a major determinant of variable IL-12p70 responses, which were also associated with numbers of circulating monocytes. Supporting the potential clinical relevance of these findings, we show that the IFN-β-IL-12p70 pathway was decreased in hospitalized COVID-19 patients and perturbed in chronically infected hepatitis C virus (HCV) patients that failed IFN-based treatment. Our study adds understanding to IL-12p70 responses in a healthy population and provides a model for dissection of variable cytokine responses for insights into disease pathogenesis.

RESULTS

TLR4 activation induces variable and stable IL-12p70 responses in a healthy population

We initially measured the secretion of 32 proteins following stimulation of whole blood with 27 stimuli and observed high levels of inter-individual variability in IL-12p70 cytokine responses to LPS and *E. coli* stimulation in 25 healthy donors of the MI cohort

(Duffy et al., 2014). To further characterize potential reasons behind this cytokine variability, we examined IL-12p70 expression by Luminex assay in 1,000 healthy donors of the MI cohort, for whom whole blood was stimulated for 22 h with LPS (TLR4 agonist) or poly (I:C) (TLR3 agonist) or left unstimulated (null control). Although an IL-12p70 response was detected for all donors upon TLR3 activation, 28% of healthy donors did not secrete detectable levels of IL-12p70 upon TLR4 stimulation (Figure 1A). As IL-12p70 is a heterodimer molecule composed of IL-12p35 and IL-12p40, the latter also forming IL-23 (with IL-12p19), we examined whether these responses showed similar variability after LPS stimulation. Measurement of IL-12p40 (Figure 1B) and IL-23 (Figure 1C) in LPS-stimulated supernatants revealed a normal distribution of responses, suggesting that the observed variability was specific for the IL-12p70 heterodimer cytokine.

To test the hypothesis that the 28% of MI donors secreted IL-12p70 concentrations below the detection limit of the Luminex assay, we developed an ultrasensitive Simoa digital ELISA. This assay has a limit of detection (LoD) of 0.01 pg/mL, which is 1,000 times more sensitive than Luminex (LoD: 18.5 pg/mL). Using this assay, we detected IL-12p70 secretion from all donors and therefore re-defined the 28% of donors that had undetectable levels of IL-12p70 with Luminex as low responders. As a group, these low responders had significantly lower levels of IL-12p70 as compared with the responders ($p < 0.0001$) (Figure 1D). To test whether this phenotype was reproducible, we measured IL-12p70 in 500 MI donors who were sampled at a second time point between 2 and 6 weeks later (visit 2; more details on specific time in the STAR Methods). The IL-12p70 response at visit 2 was also significantly different ($p < 0.0001$) between the two groups as observed in visit 1 (Figure 1E), and the IL-12p70 responses over the two time points showed a strong and significant correlation ($R = 0.71$; $p < 0.0001$; $m = 0.7292$) (Figure 1F). A two-tailed paired t test for all 500 samples with measurements from both visits was not statistically significant ($p > 0.05$).

We also confirmed that this difference was present after *E. coli* stimulation (Figure 1G) and that the IL-12p70 variance for LPS and *E. coli* ($R = 0.78$; $p < 0.0001$; $m = 1.026$) was consistent between the two visits (Figure 1H). Overall, these results indicate that inherent variability in the IL-12p70 response after TLR4 activation is a consistent and prevalent phenotype.

IL-12p70 low responders have fewer activated monocytes and dendritic cells

We next asked whether this consistently lower IL-12p70 response was due to differences in circulating immune cells. To first define the immune cells that produce IL-12p70, we stimulated whole blood of 10 healthy donors with LPS for 22 h in the presence of a protein transport inhibitor (added 8 h after stimulation initiation). A combination of 10 surface antibodies was used to characterize the major circulating immune cell populations (Table S1). As IL-23 shares the p40 subunit with IL-12p70, both IL-12p70 and IL-23 were assessed intracellularly in eight relevant cell types (gating strategy described in Figure S1A). The null condition was used to determine the positivity threshold for IL-12p70 and IL-23 secretion (Figure 2A). We observed that $CD14^+$ monocytes and dendritic cells (DCs) were the main producers of both IL-12p70 and IL-23 cytokines upon LPS stimulation (Wilcoxon test; Q value < 0.05) (Figures 2B and 2C). Interestingly, more than 30% of $CD14^+$ monocytes secreted both IL-12p70 and IL-23, whereas around 7% of $CD14^+$ monocytes secreted IL-12p70 only. A similar observation was made with conventional dendritic cells 2 (cDC2) (Figure 2D). Thus, the majority of cells producing IL-12p70 also secrete IL-23.

Taking advantage of previously published datasets (Patin et al., 2018), we examined whether the low responders of the MI cohort had fewer circulating $CD14^+$ monocytes or DCs than the responders at steady state. This immunophenotyping of the 1,000 donors was performed using 10 flow cytometry panels, from which 166 distinct immunophenotypes were reported (Patin et al., 2018). This included 76 absolute counts of circulating cells, 87 expression levels of cell surface markers, and 3 cell ratios. No significant differences between the two groups were observed for numbers of $CD14^+$ monocytes, cDC2, and plasmacytoid dendritic cells (pDCs) between low responders and responders (Figure 2E). However, an unbiased analysis applied to the entire immunophenotyping dataset revealed significantly ($Q < 0.05$) lower cell surface expression of human leukocyte antigen-DR isotype (HLA-DR) and CD86 on $CD14^+$ monocytes and HLA-DR on cDC2 in the low responders as compared with the responders (Figure 2F). No significant differences were observed regarding the cell-surface expression of CD86 on cDC2, as well as HLA-DR and CD86 expression on pDCs (Figure S1B). Taking advantage of the ultrasensitive measures provided by digital ELISA, we also examined cellular associations with IL-12p70 measures as a quantitative variable by performing linear regression on all 78 immune cell subsets (Table S2). This identified weak but significant correlations with the numbers of circulating gamma delta T cells ($R = 0.112$; $Q = 0.03$) and EMRA $CD8^+$ T cells ($R = 0.107$; $Q = 0.03$). These immune cells are classically associated with IFNγ responses and therefore may respond to IL-12p70 but do not secrete it.

Five *cis*-acting single-nucleotide polymorphisms impact IL-12p70 protein secretion, but not gene expression

Given the high prevalence of this phenotype, we next investigated for genetic associations utilizing existing genome-wide DNA datasets (Patin et al., 2018). Protein quantitative trait loci analysis (pQTL), including both *cis* and *trans*-located variants, was performed to find associations between IL-12p70 production as measured by Simoa after 22 h of LPS stimulation and 5,265,361 genotyped single-nucleotide polymorphisms (SNPs) (p value thresholds of 4.32×10^{-6} and 2.92×10^{-9} for *cis* and *trans* effects, respectively). By mapping local *cis*-acting pQTLs (within 1 Mb of the transcription start sites of *IL12A* and *IL12B* genes, which encode for the p35 and p40 subunits, respectively), we found significant associations between IL-12p70 levels and five variable genetic regions, SNPs at rs17753641, rs17809756, rs17810546, and rs76830965 and an indel at rs143060887 ($p < 4.32 \times 10^{-6}$) (Figure 3A). Donors with the homozygous major allele for these five genetic regions had a significantly higher proportion ($p < 0.0004$) of IL-12p70 low responders compared with the heterozygous genotype group. These five SNPs are all located near the *IL12A* gene

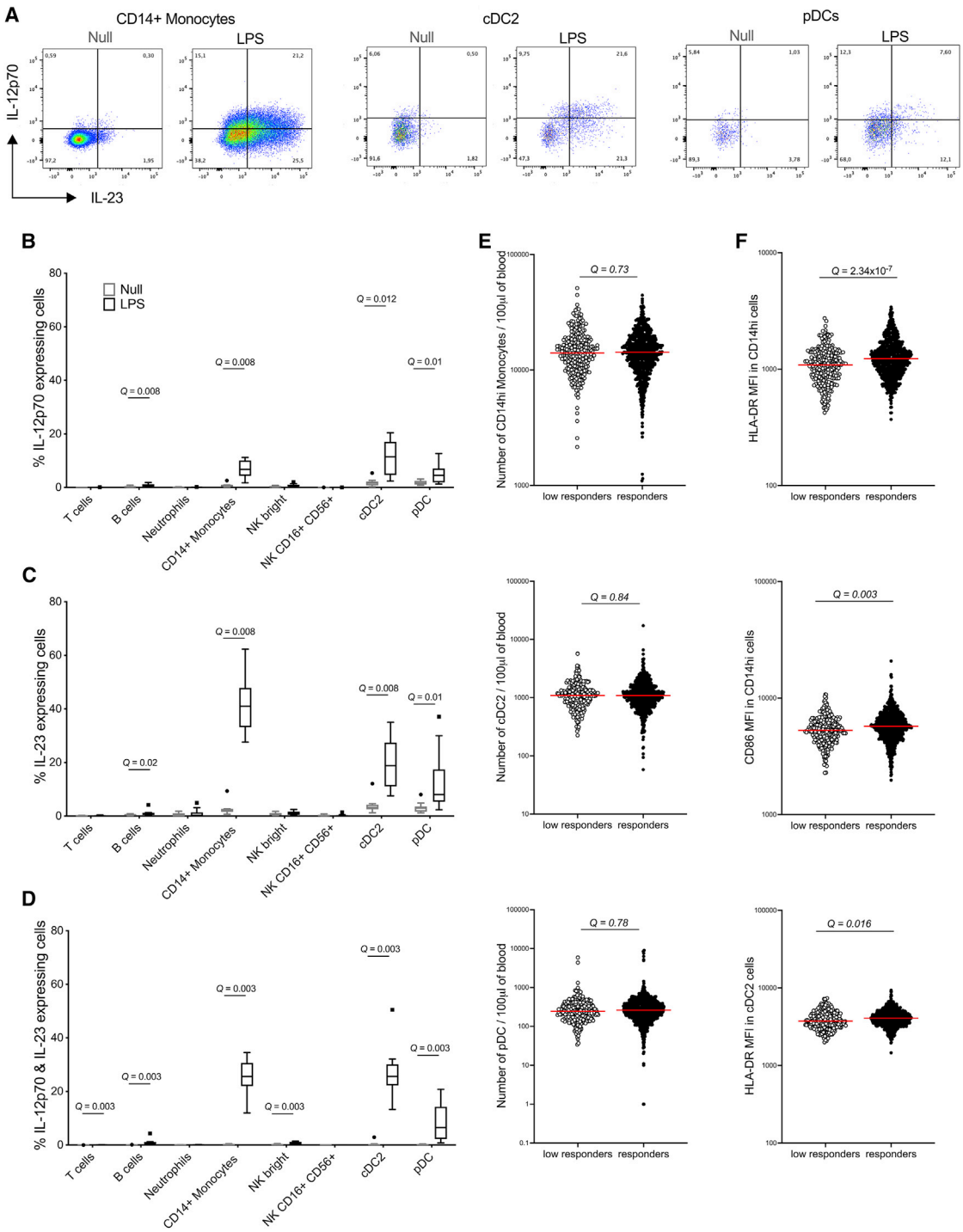


Figure 2. CD14$^+$ monocytes and dendritic cells secrete IL-12p70 after LPS stimulation

(A) Representative dot plots of IL-12p70 and IL-23 secretion upon null and LPS 22 h stimulation measured by flow cytometry in CD14$^+$ monocytes, cDC2, and pDCs.

(B–D) Tukey box-whisker plots of the percentage of IL-12p70$^+$ cells (B), IL-23$^+$ cells (C), or IL-12p70$^+$ and IL-23$^+$ cells (n = 10; D) for the indicated immune cell populations in null and LPS conditions. Q values were determined by the Wilcoxon test and false discovery rate corrected for multiple comparison testing.

(E) Numbers of CD14^{hi} monocytes, cDC2, and pDCs for the responder and low-responder groups (n = 1,000).

(F) Expression levels of HLA-DR in CD14$^+$ monocytes and cDC2 populations and CD86 in CD14$^+$ monocytes for the two groups.

Red lines indicate the median value for each group. Q values were determined by an unpaired Student's t test (on log10 values) and false discovery rate corrected for 79 cell numbers (E) and 88 cell surface protein phenotypes (F) in multiple comparison testing (n = 1,000).

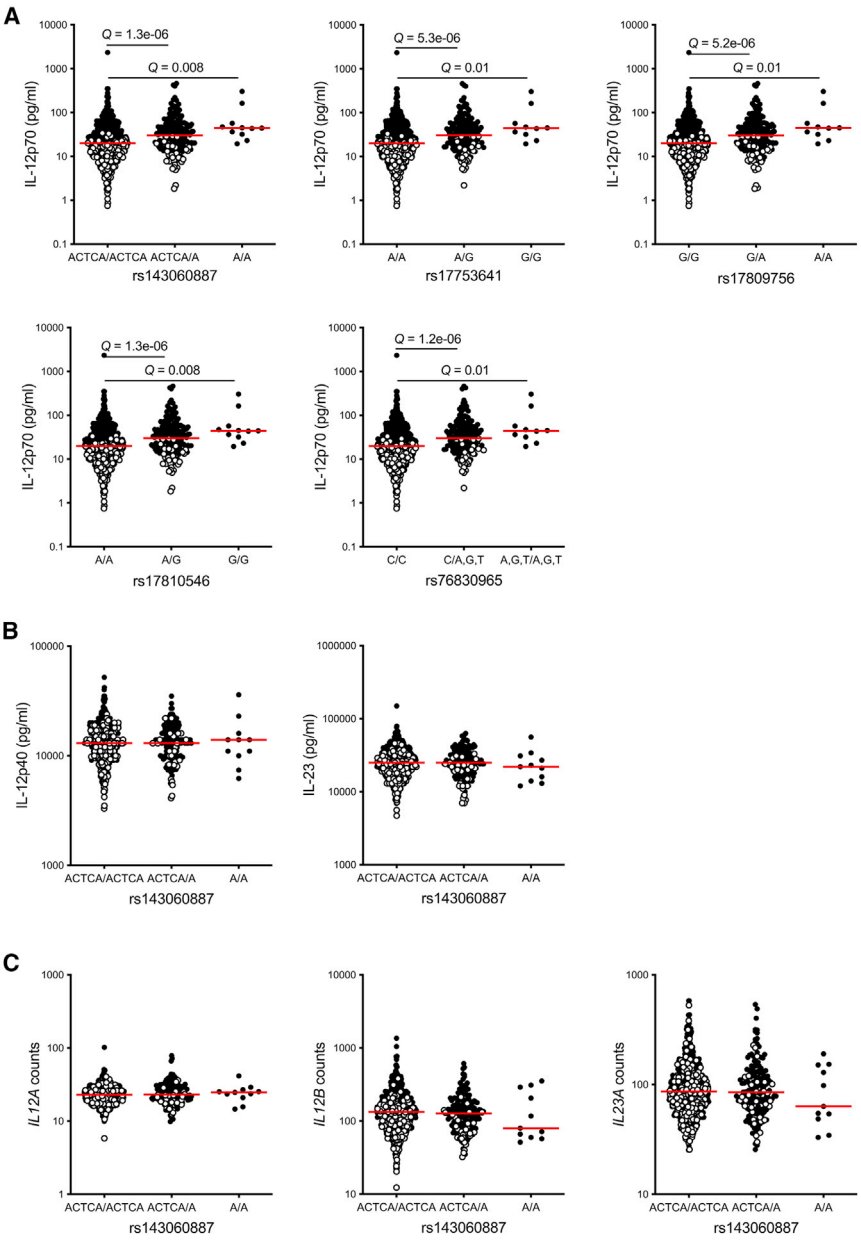


Figure 3. LPS-specific *cis*-acting pQTLs on IL-12p70 production

(A) Local pQTLs located near the *IL12A* gene acting specifically on IL-12p70 protein secretion measured by Simoa in the 1,000 *Milieu Interieur* donors in response to 22 h LPS stimulation.

(B) IL-12p40 and IL-23 proteins measured by Luminex after 22 h LPS stimulation according to the rs143060887 allelic dosage.

(C) Dot plots show rs143060887 genotype-stratified gene expression levels for the *IL12A*, *IL12B*, and *IL23A* genes measured by Nanostring after 22 h LPS stimulation.

Empty circles represent IL-12p70 low responders of the *Milieu Interieur* cohort. Red lines indicate the median value for each group. Q values were determined by an unpaired Student's t test using log10 values, and false discovery rate correction was performed to correct for multiple comparison testing.

and found to be in full or strong linkage disequilibrium in the human genome. We also performed a pQTL analysis using IL-12p70 concentrations measured in the 500 MI donors involved in the second visit and observed that these variants remained significantly associated with IL-12p70 secretion following LPS stimulation (Figure S2A). Importantly, none of these SNPs impacted IL-12p40 or IL-23 production (representative example of rs143060887 shown in Figures 3B and S2B). We thus explored whether these genetic variants impacted the gene expression of the IL-12p70, IL-12p40, and IL-23 subunits. The expression of 560 immune-related genes was measured using Nanostring for the 1,000 MI donors after 22 h of LPS stimulation. We found that the allelic dosage of the five SNPs did not impact the RNA expression levels of *IL12A*, *IL12B*, and *IL23A* genes coding for p35, p40, and p19 subunits, respectively (Figures 3C and S2C). Such an absence of the same genetic associations for induced gene and protein expression has been widely reported before (Battle et al., 2015) and may reflect kinetic or post-translational effects.

Together, our analysis indicated that the *cis*-acting variable region of IL-12A is associated with less IL-12p70 protein secretion, but not with gene expression after 22 h of LPS stimulation in healthy donors.

Variability in IL-12p70 is determined by upstream variability in IFNβ

To investigate potential molecular mechanisms behind variable IL-12p70 responses, we performed differential gene expression analysis between the responder and low responder groups. Nanostring analysis of the null condition revealed 52 differentially expressed genes (Q < 0.01) (Figure 4A; Table S3). Some of the most differential genes included HLA markers, which was consistent with the flow cytometry analysis of higher cell surface HLA-DR expression in monocytes and cDC2s (Figure 2F). Other differentially expressed genes included CIITA, MX1, and IDO1, which are related to IFNγ signaling and perhaps reflected differences in circulating effector memory cells re-expressing CD45RA (EMRA) T cells and gamma delta T cells (Table S2). However, despite highly significant statistical differences, the log2 fold changes were very modest (fold change [FC] < 0.1) (Table S3). Following LPS stimulation, 332 genes were significantly differentially expressed (Figure 4A; Table S4). We found that gene expression of the p35, p40, and p19 subunits was significantly lower (p < 0.0001) in the low

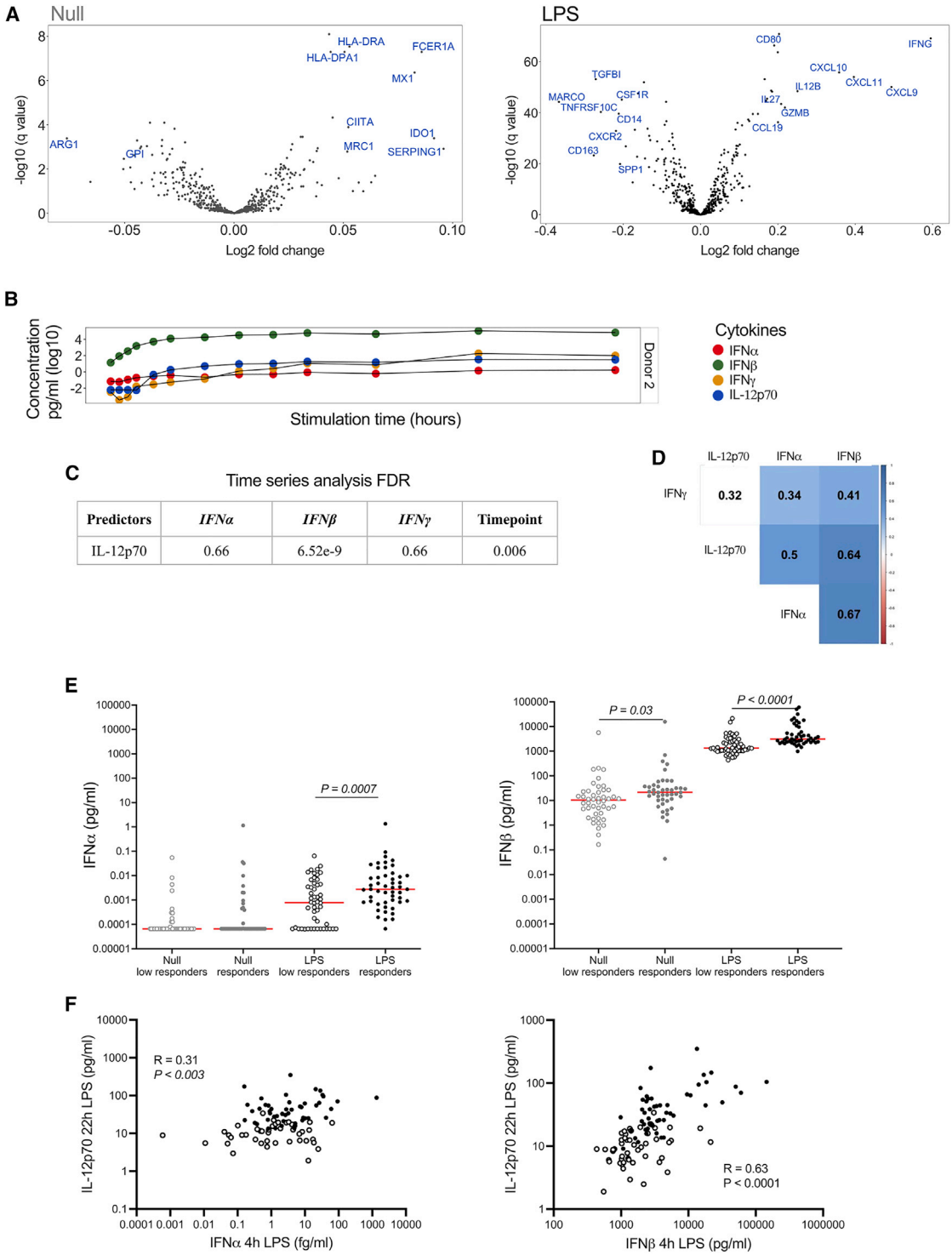


Figure 4. Kinetic responses and interactions of type I and II interferons and IL-12p70

(A) Volcano plots displaying differentially expressed genes between *Milieu Interieur* IL-12p70 low responders and responders upon 22 h null and LPS stimulation. Negative log2 fold change represents genes that are more expressed in low responders, whereas positive log2 fold change represents genes that are more expressed in responders. The cutoffs used to display gene names are Q value < 0.01, log2 fold change > 0.05 in the null stimulation condition, and log2 fold change > 0.2 in the LPS stimulation condition.

(B) Type I (IFNα and IFNβ) and II (IFNγ) interferons and IL-12p70 protein secretion over time after LPS stimulation for one healthy donor.

(C) Table reporting FDR Q values of time series analysis on protein secretion.

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responders compared with the IL-12p70 responders after LPS 22 h stimulation (Figure S3A), showing that variability in subunit transcripts may be responsible for the cytokine concentrations. IFNγ was the most differentially expressed gene with higher expression in the responders (Q = 7.34e−70 and FC = 1.51). In addition, gene set enrichment analysis showed that 74 of the 332 differentially expressed genes were involved in the IFNγ response pathway (Q = 0.02) (Figure S3B).

Previous studies reported that a first wave of IFNγ is necessary to induce IL-12p70, which triggers IFNγ secretion, further inducing IL-12p70 through a positive-feedback loop (Abdi et al., 2006; Hayes et al., 1995; Snijders et al., 1998). Moreover, type I IFNs, such as IFNα, induces *IL12A* gene expression, which codes for the p35 subunit, in response to TLR activation (Gautier et al., 2005). To assess the roles of type I and II interferons in IL-12p70 secretion upon LPS stimulation, we performed a detailed kinetic analysis. Whole blood of five healthy donors was stimulated with LPS, and IL-12p70, IFNα, IFNβ, and IFNγ were measured at 14 time points from 0 to 30 h using Simoa digital ELISA. In four out of five donors, both IFNγ and IFNα were produced either simultaneously or after IL-12p70, suggesting that these two cytokines are not responsible for IL-12p70 secretion. However, for all five donors, IFNβ secretion began 30 min to 1 h before IL-12p70 induction (a representative donor shown in Figure 4B and the other four donors in Figure S3C). These observations suggest a link between IFNβ and IL-12p70 secretion after LPS stimulation. To test this, we performed a time series analysis using a linear mixed-model approach that showed a significant association between IFNβ and IL-12p70 ($Q = 6.52 \times 10^{-9}$), whereas no significant associations were found with either IFNγ or IFNα (Q = 0.66) (Figure 4C). The Pearson correlation matrix of these kinetic data also revealed a strong association between IFNβ and IL-12p70 secretion (Figure 4D).

To determine whether the low responders of the MI cohort secreted less type I IFNs, we measured IFNα and IFNβ proteins in null and LPS TruCulture supernatants after 4 h of stimulation in a subset of 100 donors. Both IFNα and IFNβ proteins were significantly lower in the low responder group ($p = 0.0007$ and $p < 0.0001$, respectively), although IFNβ was secreted to a much higher degree than IFNα (over six logs higher and 160-fold induction for IFNβ compared with 20-fold for IFNα) (Figure 4E). Furthermore, early IFNβ production was positively and significantly correlated with later induction of IL-12p70 (Pearson's R value 0.63; $p < 0.0001$) (Figure 4F). Together, our analyses indicate that low IFNβ production is associated with the IL-12p70 low responder phenotype in whole blood of healthy donors after LPS stimulation.

The secretion of IFNβ by monocytes is essential for IL-12p70 secretion and Th1 response upon LPS stimulation

To test whether IFNβ was more essential than IFNα for LPS-induced IL-12p70 secretion, we performed additional whole-blood stimulations. Addition of either IFNα or IFNβ to LPS stimulation resulted in a significant increase in IL-12p70 (Q = 0.0034 and Q < 0.0001 respectively); however, the fold increase was a log (×9.8) greater with IFNβ (Figure 5A). Either cytokine alone did not induce IL-12p70, suggesting that type I IFNs can induce the production of only one of the IL-12p70 subunits. By contrast, the addition of IFNβ or IFNα to LPS stimulation did not increase IL-12p40 or IL-23 secretion, suggesting that type I IFNβs regulate production of the p35 subunit (Figure S4A).

To determine whether type I IFNs were essential for IL-12p70 production, we performed stimulation experiments while blocking the type I IFN pathway. Neutralization of the IFNAR1 or IFNAR2 subunits with either anti-IFNAR1 or anti-IFNAR2 monoclonal antibodies (mAbs) (neutralizing capacity validated in Figure S4B), in the presence of LPS, significantly decreased IL-12p70 secretion (Figure 5B). This result showed that signaling through the IFN type I receptor has a positive effect on IL-12p70 secretion.

We next investigated which immune cells secrete IFNβ in whole blood of healthy donors after TLR4 activation by intracellular flow cytometry (gating strategy described in Figure S1A). For all donors, we observed IFNβ secretion by $CD14^{+}$ monocytes upon LPS stimulation compared with the null condition, in which no IFNβ secretion was detected (Figures 5C, 5D, and S4C). To further confirm this result with a complementary approach, we purified five immune populations by flow-cytometry-based cell sorting after whole-blood LPS stimulation and measured IFNβ in the cell lysates. Supporting our previous results, IFNβ secretion was only detected in $CD14^{+}$ monocytes after LPS stimulation as compared with the unstimulated control (Figure 5E). This was in contrast to the response seen for IL-12p70, which was secreted by cDC2 and pDCs, as well as by monocytes. This suggests that the initial early IFNβ response is dependent on monocytes, from which additional innate immune cells are triggered to respond with downstream cytokine responses. Correlation analysis between LPS-induced IFNβ with the available 78 counts of circulating cells in the 1,000 MI donors supported these results, with both $CD14^{hi}$ and $CD16^{hi}$ monocytes showing the most significant associations ($Q < 0.002$) with IFNβ responses (Table S5).

To provide additional insight into variability of the IFNβ response, we performed a *cis*- and *trans*-pQTL analysis after 4 h of LPS stimulation in MI donors. We found significant associations between four genetic variants rs10757182, rs10811473, rs13291085, and rs7020908 and IFNβ levels ($p < 4.32 \times 10^{-6}$) (Figure 5F). These SNPs are in strong linkage disequilibrium (LD) (r^2 0.97–1) and are located within 1 Mb of the *IFNB1* gene coding for IFNβ. For all SNPs, no significant differences were observed regarding the proportion of IL-12p70 low responders among the three allelic dosages of each variant and none of the variants impacted *IFNB1* gene expression measured at this time point (Figure S4D).

(D) Pearson correlation matrix between cytokine responses.
(E) Type I IFNs measured by Simoa after 4 h LPS stimulation of whole blood for the low responders and responders. The red lines indicate the median value for each group and stimulation condition. p values were determined by the unpaired Student's t test; n = 100 donors.
(F) Correlation between IFNα or IFNβ secretion after 4 h of LPS stimulation and IL-12p70 secretion upon 22 h LPS stimulation. Pearson correlation tests were performed on log10 values; n = 100 donors. Empty circles represent IL-12p70 low responders of the *Milieu Interieur* cohort.

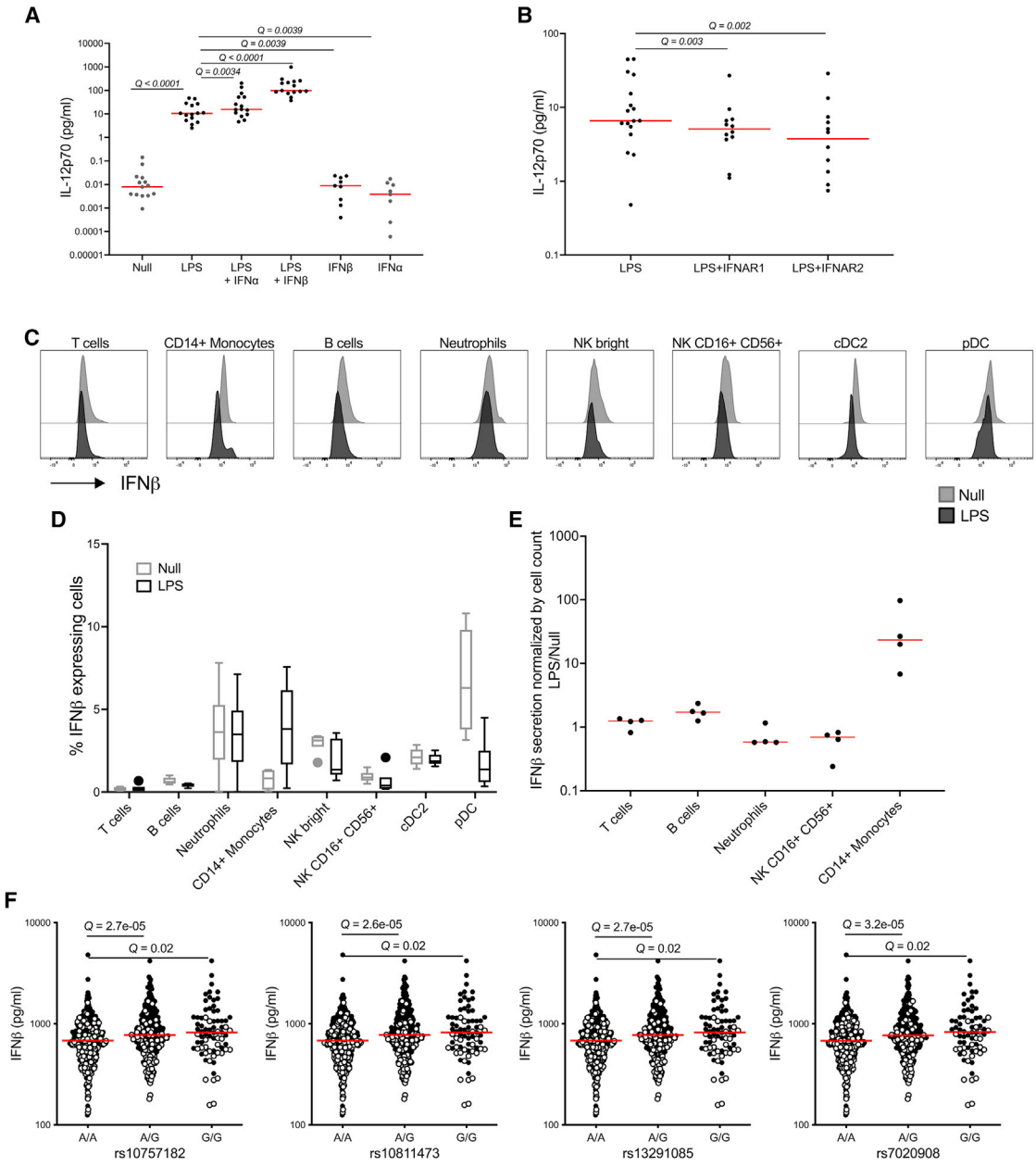


Figure 5. Impact of LPS, type I IFNs, and IFNAR inhibitors on IL-12p70 production

(A) IL-12p70 secretion measured by Simoa in whole blood after 22 h stimulation with LPS, type I IFNs, the combination of LPS and type I IFNs (n = 9), and an unstimulated control (null). The red lines indicate the median value for each stimulation condition, Q values were determined using the Wilcoxon test, and false discovery rate correction was performed to correct for multiple comparison testing.

(B) IL-12p70 secretion measured by Simoa in whole blood after 22 h stimulation with LPS, LPS with IFNAR neutralizing antibodies. The red lines indicate the median value for each stimulation condition, Q values were determined using the Wilcoxon test, and false discovery rate correction was performed to correct for multiple comparison testing.

(C) Representative histograms showing intracellular IFNβ production measured by flow cytometry in T cells, $CD14^{+}$ monocytes, B cells, neutrophils, NK bright cells, NK cells, cDC2, and pDC populations after 6 h of null or LPS whole-blood stimulation.

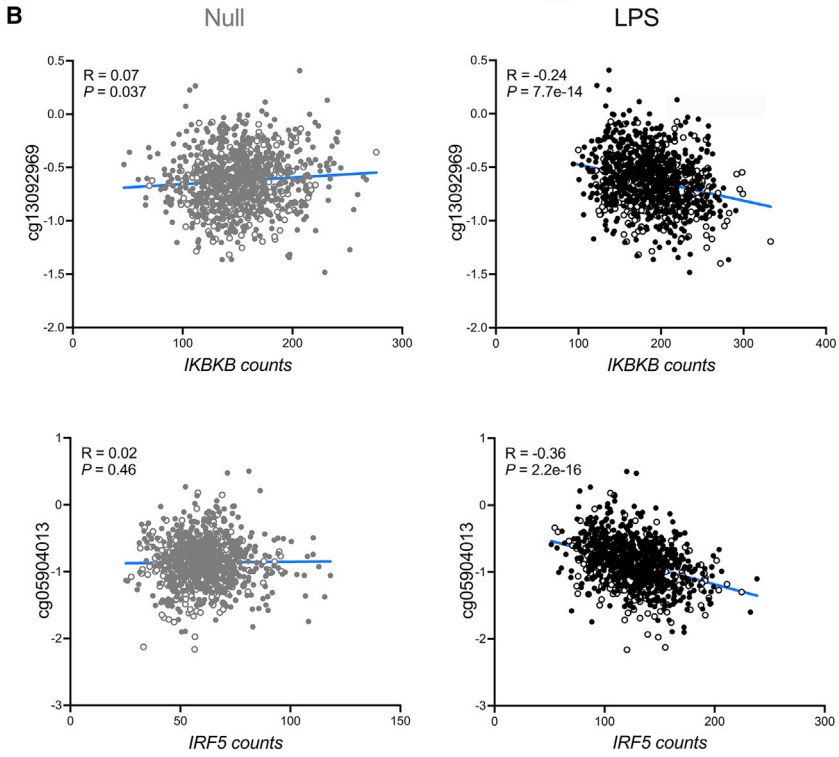
(D) Tukey box-whisker plots of the percentage of IFNβ cytokine-positive cells for the indicated immune cell populations in null and LPS conditions; n = 6.

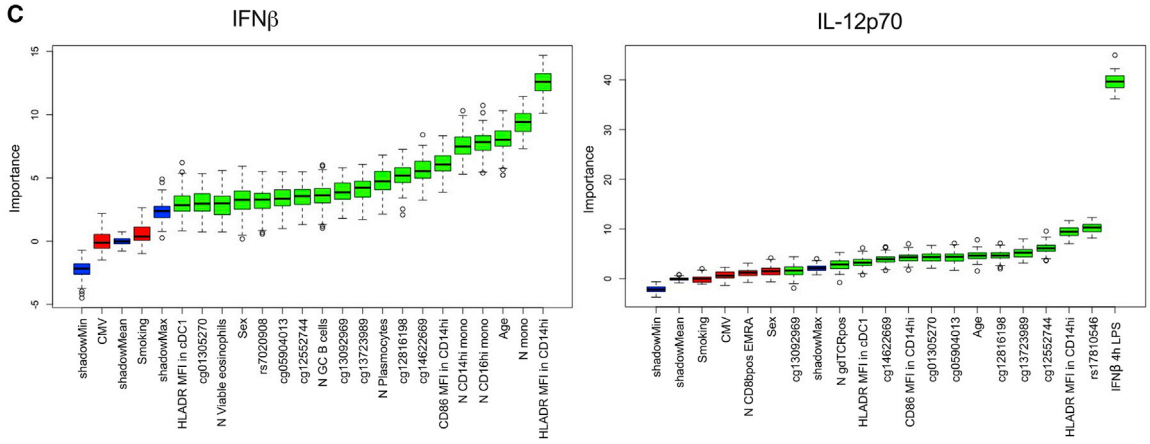
(E) Intracellular IFNβ (LPS/null ratio) measured by Simoa after 3 h null and LPS whole-blood stimulation, cell sorting, and lysis of purified cells; n = 4 with a minimum of 100,000 purified cells per population.

(F) *cis*-acting variants significantly associated with LPS-induced IFNβ protein secretion measured in the 1,000 *Milieu Interieur* donors after 22 h LPS stimulation.

A

Probe	Chromosome	Probe position	Gene	Q value	Highest methylation group
cg12816198	7	128577593	*IRF5*	0,016	responders
cg12552744	8	42127692	*IKBKB*	0,016	responders
cg13723989	8	42127640	*IKBKB*	0,021	responders
cg01305270	8	42174685	*IKBKB*	0,021	responders
cg05904013	7	128579933	*IRF5*	0,025	responders
cg14622669	7	128577606	*IRF5*	0,035	responders
cg13092969	8	42129644	*IKBKB*	0,039	responders





(legend on next page)

We demonstrated that IFNβ has a stronger synergistic effect than IFNα for inducing IL-12p70 secretion after LPS stimulation and is mainly produced by monocytes in whole blood. In addition, the production of IFNβ protein is associated with a *cis*-acting variable genetic region.

Identification of epigenetic contributors to IL-12p70 variability and inclusion in an integrative model of cytokine variability

As epigenetic changes can drive cytokine secretion and control the level of immune responses (Morandini et al., 2016), we next assessed whether DNA methylation contributes to IL-12p70 secretion variability, utilizing gene methylation datasets from MI donors. To maintain sufficient statistical power, we focused our analysis on 49 selected genes involved in the LPS response pathway (listed in Table S6). Of these selected genes, we identified 29 probes (out of 1,215 probes tested), corresponding to 20 genes that were well distributed across the genes of interest (Table S7; Figure S5A), that showed significantly different methylation levels between the IL-12p70 low responders and responders ($Q < 0.05$). Interestingly, seven probes that showed increased methylation in the responder group were associated with *IKBKB* and *IRF5* genes (Figure 6A) and five probes with TRAF3 and TRAF6 (Table S7; Figure S5B). The *IKBKB* gene codes for the IKKb kinase, which phosphorylates and activates the transcription factor IRF5 (Ren et al., 2014), which plays a role in inducing IFNβ expression (Bergstrøm et al., 2015; del Fresno et al., 2013). The methylation levels of these probes negatively correlated with *IKBKB* and *IRF5* gene expression in the LPS stimulation condition, but not in the null control (example shown in Figure 6B). TRAF3 and TRAF6 are implicated in nuclear factor κB (NF-κB)-mediated signaling, but only two probes in TRAF3 (and none of the TRAF6 probes) showed negative associations with their corresponding gene expression after LPS stimulation (Figure S5B). These results highlight the impact of methylation on *IKBKB* and *IRF5* gene expression for downstream cytokine responses to LPS.

To assess the relative importance of the intrinsic and genetic factors identified in our different analyses, we utilized the feature selection algorithm Boruta (Kursa and Rudnicki, 2010). Based on previous studies highlighting the importance of age (Carr et al., 2016), sex (Giefing-Kröll et al., 2015), smoking (Qiu et al., 2017), and cytomegalovirus (CMV) status (Sylwester et al., 2005) on immune variability, these were included in the model along with the biological factors identified above. While CMV status and smoking showed no importance for IFNβ or IL-12p70 cytokines, sex appeared to have importance for IFNβ, but not IL-12p70, secretion (Figure 6C). IFNβ secretion, genetic variants, and HLA-DR mean fluorescence intensity (MFI) in monocytes were the top three variables showing high importance for variable IL-12p70 secretion, whereas HLA-DR MFI in monocytes and age were particularly important for IFNβ secretion. To retain some potential nonlinear effects, selected features from the Boruta algorithm were integrated into a conditional inference tree to build a regression model between IFNβ, or IL-12p70, secretion and the genetic and nongenetic independent variables (Figure S5C). This approach showed that age effects on IFNβ secretion were relevant for donors with lower numbers of monocytes and lower HLA-DR expression (Figure S5C), perhaps reflecting the overall decline of monocytes with age, as we previously reported for this cohort (Patin et al., 2018). For IL-12p70, the conditional inference tree was more dispersed and, in this case, showed that HLA-DR monocyte expression was more relevant in donors with higher IFNβ secretion (Figure S5C). It also showed potential differential effects of both SNPs and methylation effects, depending on cytokine concentrations (Figure S5C).

Validation of the IFNβ/IL-12p70 pathway in acute and chronic viral infection

To determine whether the IL-12p70 response is also associated with IFNβ secretion in disease settings, we first assessed the secretion of IFNβ and IL-12p70 cytokines in mild, hospitalized, and convalescent COVID-19 patients. An impaired type I IFN activity was previously described in severe COVID-19 patients (Hadjadj et al., 2020), highlighting the relevance of studying this pathway in this disease. Patients were identified and recruited as described in the STAR Methods, and whole blood was stimulated for 22 h with LPS and an unstimulated control (null) using the same TruCulture system as applied to MI donors. IFNβ and IL-12p70 responses were measured in the TruCulture supernatants by Simoa digital ELISA. We observed a significant decrease of both IFNβ and IL-12p70 cytokines in hospitalized patients compared with the healthy, mild, and convalescent groups following whole-blood LPS stimulation (Figure 7A), but not in the null condition (Figure S6A). IL-12p70 secretion was positively and significantly correlated with IFNβ secretion in all groups of patients (Figure 7B). This result reveals a decreased activation of the IFNβ/IL-12p70 axis after TLR4 activation in patients hospitalized from COVID-19 infection, a phenotype that was absent after patient recovery.

To further investigate the IFNβ/IL-12p70 pathway in disease, we next examined secretion of IFNβ and IL-12p70 in chronic viral infection. HCV was selected for this, as a type I IFN gene signature has been previously implicated in immunopathogenesis (Sarasin-Filipowicz et al., 2008), and the patients were treated with 6 months of pegylated IFN plus ribavirin (the standard-of-care treatment at the time of the clinical study). One hypothesis for these treatment failures is IFN desensitization (Sarasin-Filipowicz et al., 2008), thus providing an opportunity to test the applicability of our findings in a relevant clinical context. Patients were identified and recruited as described in the STAR Methods, and

Figure 6. Differential methylation probes between low and high responders and integrative modeling of IFNβ and IL-12p70 variability

(A) List of *IKBKB* and *IRF5* gene methylation probes significantly different between the *Milieu Interieur* low responders and responders.

(B) Correlation of one IKBKB and IRF5 probe methylation values with their respective gene expression measured by Nanostring after null and LPS 22 h stimulations. Pearson correlations were performed using gene expression log10 values. Empty circles represent IL-12p70 low responders of the *Milieu Interieur* cohort.

(C) IFNβ and IL-12p70 Boruta variable importance charts, including all genetic and non-heritable factors selected from the exploratory analysis as well as age, sex, CMV status, and smoking. Red and green box plots represent *Z* scores of rejected and confirmed attributes, respectively. Blue box plots correspond to minimal, average, and maximum *Z* scores of a shadow attribute (n = 1,000).

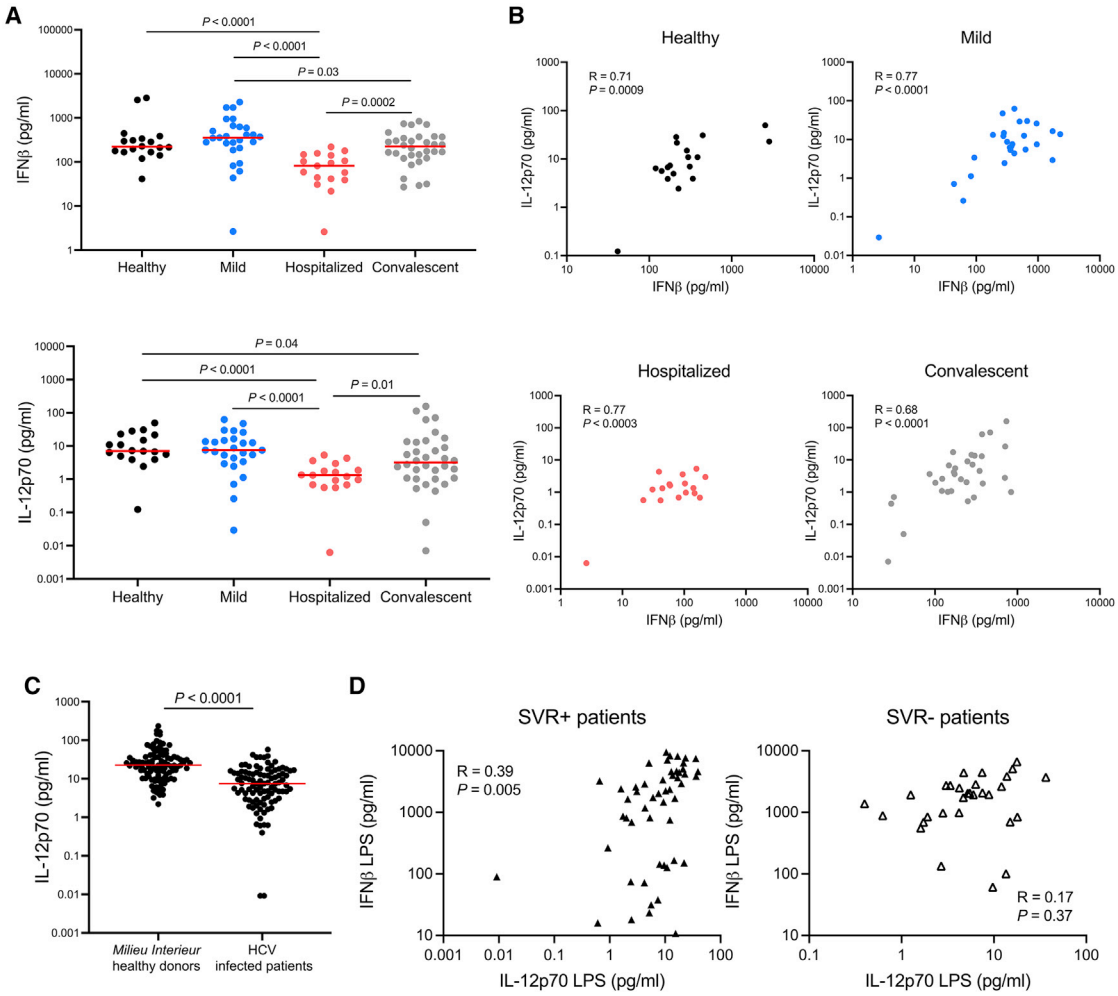


Figure 7. IFNβ secretion drives IL-12p70 variability in healthy donors and viral infection

(A) IFNβ and IL-12p70 measured using Simoa after stimulation of whole blood with LPS for 22 h from healthy controls (n = 18) and patients acutely infected with SARS-CoV-2 presenting mild symptoms (n = 29) or being hospitalized for moderate, severe, or critical symptoms (n = 18) or convalescent patients (n = 34). p values were determined by Mann-Whitney tests, and the red lines indicate the median value for each severity condition.

(B) Correlation between IFNβ and IL-12p70 secretion upon LPS stimulation in healthy controls (n = 18) or mild (n = 29), hospitalized (n = 18), or convalescent (n = 34) COVID-19 patients. Pearson correlations were performed using cytokine concentration log10 values.

(C) IL-12p70 measured using Simoa after stimulation of whole blood with LPS for 22 h from healthy donors (n = 100) and patients infected with HCV (n = 100). p values were determined by Mann-Whitney tests, and red lines indicate the median value.

(D) Correlation between IFNβ and IL-12p70 secretion upon LPS stimulation in SVR^{-} HCV patients (n = 33 patients) and in SVR^{+} HCV patients (n = 54 donors). Pearson correlation tests were performed on log10 values.

whole blood was stimulated for 22 h with LPS and an unstimulated control (null) using the same TruCulture system as applied to MI donors, after which we measured IFNβ and IL-12p70 responses by Simoa digital ELISA. IL-12p70 cytokine secretion measured with the Simoa assay was significantly lower ($p < 0.0001$) in patients infected with HCV than in healthy donors following LPS stimulation, suggesting that the Th1 response is altered in this disease (Figure 7C). Patients were divided into those that were sustained virological responders (SVR^{+}) or not (SVR^{-}) after 6 months of treatment with pegylated IFN plus ribavirin. No significant differences were observed in IL-12p70 or IFNβ secretion between the SVR^{+} and SVR^{-} patients for any of the stimulation conditions (Figure S6B). IFNβ positively and significantly correlated with IL-12p70 secretion after LPS stimulation in SVR^{+} patients (Pearson's R value 0.39; $p = 0.005$), but not in the SVR^{-} patients (Pearson's R value 0.17; $p = 0.37$) (Figure 7D). However, additional analysis including all HCV patients with an interaction term between cytokine responses and SVR did not identify a significant association. These results show that the IFNβ-IL-12p70 pathway is altered in acute and chronic viral infection but is resolved after viral clearance.

DISCUSSION

In this study, we dissected natural variability in IL-12p70 responses by applying a systems immunology approach to a

population-based study. We characterized a consistently low IL-12p70 response upon LPS whole-blood stimulation in a quarter of a healthy population cohort, which allowed us to classify subjects into two groups regarding their immune responses to TLR4 activation. We also demonstrated that upstream IFNAR signaling has a synergistic effect on immune responses to bacteria consistent with previous studies (Gautier et al., 2005) but identified IFNβ as the predominant IFN subtype for optimal IL-12p70 secretion. Previous work has shown that IL-12p70 positively regulates its own production via IFNγ, which primes monocytes and neutrophils to activate IFN-regulatory factors inducing the transcription of both *IL12A* and *IL12B* genes (Hayes et al., 1995; Ma et al., 1996). The ability of IFNγ to enhance production of IL-12p70 creates a positive-feedback mechanism during inflammation and Th1 responses. Our results were in support of these previous studies but highlighted an important early role for IFNβ after LPS stimulation.

This finding highlights the importance of integrating upstream cytokine networks for understanding variability in immune responses. While we identified both genetic and epigenetic effects on inter-individual variance for both cytokine responses, they were not the main determinant, as previously reported (Li et al., 2016b). Here, we found that levels of monocyte HLA-DR and CD86 cell surface expression on circulating immune cells had a similar impact on induced IFNβ response variability as the combined effects of genetic and epigenetic factors. These results were also supported by the finding of lower expression of HLA-DR genes, suggesting a qualitative rather than a quantitative cellular effect on induced cytokine responses. Low expression of HLA-DR and CD86 have been previously associated with impaired cellular functions of monocytes, including reduced pro-inflammatory properties and a decreased ability to induce T cell responses through antigen presentation (Astiz et al., 1996; Piani et al., 2000). Thus, our results suggest that low responders may have an increased incidence or be at greater risk of bacterial infection. The importance of the IL-12p70-IFNγ axis in bacterial infection has been clearly demonstrated by previous studies of Mendelian susceptibility to mycobacterial infection (MSDM) (Martínez-Barricarte et al., 2018). As clinical examples of MSDM are extremely rare, our study shows how such information can be obtained at the population level to identify lower impact but higher frequency genetic variants that likely combine with other factors for an overall increased disease risk.

Highlighting the strength of our approach, we report two independent genetic associations with both IL-12p70 and IFNβ protein secretion specific to LPS stimulation. All pQTLs described are *cis*-QTLs, suggesting that these pQTLs directly influence cytokine secretion, contrary to recent reports that identified *trans*-QTLs as having a stronger indirect influence on cytokine secretion (Li et al., 2016a, 2016b). We (Quach et al., 2016) and others (Rink et al., 1998; Fairfax et al., 2014) have previously reported an SNP (rs12553564) that associates with variable IFNβ gene expression in LPS-stimulated monocytes that has a downstream *trans*-effect on multiple immune genes. Surprisingly, this SNP was not significantly associated with either IFNβ or IL-12p70 secretion in our study. This difference may be explained by the measurement of secreted proteins rather than RNA or by the multi-cellular composition of blood analyzed, but clarifying this will require further studies.

The effects of age and sex on IFNβ and IL-12p70 production were relatively moderate. We observed that age had a higher impact on variance of both cytokines as compared with sex, suggesting that aging or environmental exposures may affect cytokine secretion more than sex. Variation in Th1 immune responses due to age has also been reported by Ter Horst et al. (2016), who showed a significant decrease of IFNγ with age following whole-blood LPS stimulation. Our results suggest that Th1 cytokine production is impacted by immunosenescence, as previously documented (Crooke et al., 2019; Rink et al., 1998).

Limitations of the study

Our study also presents some limitations. First, we cannot exclude the possibility that the donors with low IL-12p70 had not been exposed to low-level environmental LPS or other infectious agents prior to blood collection (Kox et al., 2011; Rittig et al., 2015) or experienced sub-clinical levels of inflammation. This hypothesis may be supported by the weak though significant higher levels of baseline genes associated with IFNγ responses, as well as elevated T cell subsets, and may be an avenue for further investigation. A second limitation was that gene expression was measured for 560 immune genes, which does not provide an unbiased view of the transcriptome. In addition, the genotyping method used did not provide extensive information about rare genetic variants and structural variation. Thus, our systems immunology approach may be extended in future studies using RNA sequencing and whole-genome sequencing to address these limitations. Third, the dose of LPS used to stimulate whole blood (10 ng/mL) is higher than the dose that induces sepsis (Fullerton et al., 2016). Although selected by titration experiments to be in the biological response range (Duffy et al., 2014), this relatively high dose induces a strong cytokine response that may mask potential contributions of genetic and non-heritable determinants. As a consequence, the IFNβ/IL-12p70 observed variability may not fully reflect natural variability present during bacterial infection. Finally, a non-negligible part of IL-12p70 and IFNβ immune response variance remains unexplained by our models (~67% and ~88%, respectively). Thus, additional investigations are required to assess the contribution of other determinants, more specifically the impact of environmental exposures; the effects of additional genetic control, including unexplored transcripts and indels; and alternative epigenetic effects, such as chromatin remodeling.

Alternatively, we may also consider that such biological systems are inherently noisy and that, despite best efforts to standardize assays and integrate multiple levels of variability, we may not be able to explain and account for all variability (Eling et al., 2019). However, the application of our findings to HCV and COVID-19 patients shows that even an uncomplete understanding of variable immunity may be useful for a better understanding in disease. Notably, the immune perturbations that we observed in acute infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were absent in recovered convalescent patients but were present in chronic HCV infection, suggesting that viral infection directly impacts this pathway.

Chronic HCV viral infection has been shown to have a long-term impact on certain immune phenotypes, even after successful viral clearance (Alanio et al., 2015; Hengst et al., 2016), and our observation may also help to explain some of the increased risk for bacterial co-infection during ongoing viral infection.

Overall, our study provides findings on how IL-12p70 is produced and regulated against bacterial stimulation both in health and disease settings and a better understanding of how intrinsic and genetic factors drive inter-individual immune responses. The characterization of the determinants of cytokine variation in healthy subjects is essential to identify causes that lead to aberrant cytokine secretion in immune-related disorders for development of potential treatments. The systems immunology model described here allows the dissection of such complex pathways in a population setting for direct relevance to human studies and clinical applications.

CONSORTIA

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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 can be found online at https://doi.org/10.1016/j.celrep.2022.110989.

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AUTHOR CONTRIBUTIONS

C.P. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. A.L. performed specific experiments and analysis and interpreted data. B.C., J.Boussier, V.B., and N.S. performed experiments. V.R., J.Bergstedt, V.S.-A., M.S.K., M.R., and E.P. analyzed data. L.T., J.A.S., C.N.C., N.C., E.M., and S.P. collected patient samples and analyzed data from clinical studies. M.L.A., L.Q.-M., and D.D. conceived the study, obtained funding, and provided guidance. D.D. designed and supervised the whole study, designed experiments, interpreted data, and wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

DECLARATION OF INTERESTS

M.L.A. is a current employee of Hibio, who had no influence on the study design or reporting. D.D. declares previous grant support from Rules Based Medicine that was not implicated in this study. The other authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure gender balance in the recruitment of human subjects. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-pan-IFN α (capture)	ImmunoQure	8H1 clone
Anti-pan-IFN α (detector)	ImmunoQure	12H5 clone
Anti-IFNβ (capture)	PBL	ref. 710322-9
Anti-IFNβ (detector)	PBL	ref. 710323-9
Anti-IFN γ (capture)	Biolegend	ref. 507501
Anti-IFN γ (detector)	R&D	ref. MAB285; RRID:AB_2123306
Anti-IFNβAR1	MedImmune	Anifrolumab; RRID:AB_2911153
Anti-IFNβAR2	PBL	Clone MMHAR-2; RRID:AB_212039
Anti-CD3	BD	ref. 560176; RRID:AB_1645475
Anti-CD19	BD	ref. 563036; RRID:AB_2737968
Anti-CD56	BD	ref. 557747; RRID:AB_396853
Anti-CD14	BD	ref. 561391; RRID:AB_10611856
Anti-CD16	BD	ref. 562293; RRID:AB_11151916
Anti-CD66b	BD	ref. 562940; RRID:AB_2737906
Anti-HLA-DR	BD	ref. 339216; RRID:AB_2868719
Anti-BDCA1 (CD1c)	BD	ref. 742751; RRID:AB_2741017
Anti-BDCA2 (CD303)	BioLegend	ref. 354228
Anti-BDCA4 (CD304)	BD	ref. 743130; RRID:AB_2741297
Anti-IL-12p70	Miltenyi	ref. 130-103-673; RRID:AB_2652340
Anti-IL-23p19	eBioscience	ref. 12-7823-42
Bacterial and virus strains		
HK *E. coli* 0111:B4	Invivogen	tlrl-hkeb2
LPS-EB (ultrapure)	Invivogen	tlrl-eblps
Poly I:C	Invivogen	vac-pic
Biological samples		
Milieu Interieur blood samples	Milieu Interieur cohort (Institut Pasteur)	NCT03905993
COVID-19 patient blood samples	STTAR-Bioresource of TCD-SJH-TUH	REC 2020-03
HCV patient blood samples	ANRS/INSERM	C10-08
Healthy donor blood samples	CoSImmGEn cohort (Institut Pasteur)	NCT03925272
Chemicals, peptides, and recombinant proteins		
IFNα	PBL	ref. 11150
IFNβ	PBL	ref. 11415-1
IFNγ	PBL	ref. 11500
Trizol LS	Thermofisher	ref. 10296010
Critical commercial assays		
Nanostring Human Immunology nCounter V2 panel	Nanostring	N/A
RBM Human Core 1 and Core 2 Luminex panels	Rules Based Medicine	N/A
Deposited Data		
Genotyping data	Milieu Interieur	EGA (EGAS00001002460)
Methylation data	Milieu Interieur	https://doi.org/10.1101/2021.06.23.449602
Cytokine data	This manuscript	Data S1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Boruta package of R	Kursa and Rudnicki (2010)	https://www.rdocumentation.org/packages/Boruta/versions/7.0.0/topics/Boruta
Caret package in R	Kursa and Rudnicki (2010)	https://cran.r-project.org/web/packages/caret/vignettes/caret.html
ctree function of the Party package in R	Hothorn et al. (2006)	https://www.rdocumentation.org/packages/partykit/versions/1.2-15/topics/ctree
R package "broom"' v0.5.2	https://github.com/dgrtwo/broom_paper	https://cran.r-project.org/web/packages/broom/index.html

RESOURCE AVAILABILITY

Lead contact

- Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Darragh Duffy (darragh.duffy@pasteur.fr).

Materials availability

No materials were developed in the context of this study.

Data and code availability

- Data availability

Genetic data used in this study is available at EGA (EGAS00001002460), methylation data has been described in an additional study (Bergstedt et al., 2021) and will also be deposited at EGA upon publication. Cytokine and gene expression source data are provided with this paper in Data S1.

- Code availability

This paper does not report original code.

- Software and algorithms

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

The Milieu Interieur cohort

The 1,000 healthy donors of the *Milieu Interieur* cohort were recruited by BioTrial (Rennes, France) from September 2012 to July 2013. It is composed of 500 women and 500 men, including 200 individuals per decade of life, between 20 and 69 years of age. The donors were selected based on inclusion and exclusion criteria detailed elsewhere (Thomas et al., 2015). The recruitment was restricted to individuals from metropolitan French origin for three generations. Donors were defined as healthy according to medical history, clinical examination, laboratory results, and electrocardiography. General information about socio-demographic, health-related life habits, childhood disease, vaccination history and family health history were reported in an electronic case report form (eCRF). Half of the subjects were randomly selected (stratified by age and sex) to return for a visit 2 that took place between 2 to 6 weeks after visit 1 (median 16 days, range 14-44 days). The clinical study was approved by the Comité de Protection des Personnes - Ouest 6 on June 13, 2012, and by the French Agence Nationale de Sécurité du Médicament on June 22, 2012, and was performed in accordance with the Declaration of Helsinki. The study was sponsored by the Institut Pasteur (Pasteur ID-RCB Number 2012-A00238- 35) and conducted as a single-center study without any investigational product. The original protocol is registered under ClinicalTrials.gov (NCT01699893). Informed consent was obtained from the participants after the nature and possible consequences of the studies were explained. The samples and data used in this study were formally established as the *Milieu Interieur* biocollection (NCT03905993), with approvals by the Comité de Protection des Personnes – Sud Méditerranée and the Commission nationale de l'informatique et des libertés (CNIL) on April 11, 2018.

Hepatitis C cohort

HCV patients were sampled as part of a prospective study (C10-08) sponsored by the Institut National de la Sante et de la Recherche Medicale (Inserm) and by the Agence Nationale de Recherche sur le Sida et les Hepatites Virales (ANRS-C10-08). Approval of the

study was obtained from the French Comite de protection des personnes (CPP IDF II). Patients infected with hepatitis C virus were recruited at four different hospitals/centers in Paris Ile-de-France, France. The cohort included 100 donors from 18 to 70 years old infected with genotype 1 or 4 of the virus. Blood of the hepatitis C infected patients was collected prior to interferon treatment initiation. Written informed consent was obtained from all study participants.

COVID-19 cohort

Healthy controls and patients acutely infected with SARS-CoV-2 virus were recruited as inpatients or as outpatients following receipt of a positive SARS-CoV-2 nasopharyngeal swab PCR test at St James's Hospital (SJH) in Dublin, Ireland. Ethical approval was obtained for the study from the Tallaght University Hospital (TUH)/St James's Hospital (SJH) Joint Research Ethics Committee (reference REC 2020-03). The cohort included 18 healthy donors, 29 patients presenting mild symptoms, 18 hospitalized patients presenting moderate, severe or critical symptoms from 21 to 91 years old. Matched convalescent samples for 34 donors were collected as part of post-COVID-19 outpatient clinic. Written informed consent was obtained from all study participants.

Healthy human fresh whole blood

For experimental validation, fresh whole blood was collected into sodium heparin tubes from healthy French volunteers enrolled at the Clinical Investigation and Access to BioResources (ICAReB) platform (Center for Translational Research, Institut Pasteur, Paris, France). These donors were part of the CoSImmGEn cohort (NCT03925272). Written informed consent was obtained from all study participants.

METHOD DETAILS

Immunophenotyping

Ten flow cytometry panels of eight-colors each were developed in order to enumerate and phenotype the major circulating leukocyte populations of the 1,000 *Milieu Interieur* donors. Premix of antibodies were manually made and staining protocol was performed using the Freedom Evo 150 liquid handling system (Tecan). Samples were acquired using MACSQuant analyzers. Panel antibodies and gating strategies were done as previously reported (Hasan et al., 2015). Converted FCS format files of 313 immunophenotypes were analyzed using FlowJo software version 9.5.3, from which a total of 166 flow cytometry measures were retained including 87 MFI, 76 cell counts and 3 cell ratios (Patin et al., 2018).

Genotyping

DNA genotyping of 719,665 SNPs was performed using the HumanOmniExpress-24 BeadChip (Illumina) for the 1,000 *Milieu Interieur* donors. To improve coverage of rare SNPs, 966 of the 1,000 donors were also genotyped at 245,766 exonic SNPs using the HumanExome-12 BeadChip (Illumina). After quality control filters previously described, the final data set included 5,265,361 QC-filtered SNPs (Bergstedt et al., 2021).

Whole genome DNA methylation profiling

Genomic DNA of the 1,000 *Milieu Interieur* donors was extracted and treated with sodium bisulfite (Zymo Research) and bisulfite-converted DNA was applied to the Infinium MethylationEPIC BeadChip (Illumina, California, USA), using the manufacturer's standard conditions. The MethylationEPIC BeadChip measures single-CpG resolution DNAm levels at 866,836 CpG sites in the human genome. DNA methylation data were analyzed as described (Bergstedt et al., 2021).

Whole blood TruCulture stimulation

TruCulture tubes (Myriad RBM) containing LPS-EB (ultrapure) (10ng/mL), heat killed *E. coli* 0111:B4 (10^7 bacteria), and Poly I:C (20ug/mL) (all Invivogen) dissolved in 2mL of buffered media were batch produced as previously described. Tubes were thawed at room temperature and 1mL of fresh blood was distributed into each tube within 15 min of collection. Tubes were mixed by inverting them and incubated at 37°C (±1°C) for 4 or 22 h in a dry block incubator. After the incubation time, a valve was manually inserted into the tube to separate the supernatant from the cells. Supernatant was collected, aliquoted and immediately stored at −80°C for protein secretion analysis. Cell pellets of the TruCulture tubes were resuspended in 2mL of Trizol LS (Sigma) and tubes were vortexed for 2 min at 2000 rpm and stored at −80°C for gene expression analysis. The kinetic study of gene expression and cytokines was previously described (Bisiaux et al., 2017).

Gene expression analysis

Total RNA was extracted from the null and LPS TruCulture cell pellets for the 1,000 *Milieu Interieur* donors using NucleoSpin 96 miRNA kit (Macherey-Nagel). RNA concentrations were measured using Quantifluor RNA system kit (Promega) and RNA integrity numbers were determined using the Agilent RNA 6000 Nano kit (Agilent Technologies). Total RNA samples were analyzed using the Human Immunology v2 panel profiling 594 immunology-related human genes (Nanostring). Gene expression data were normalized as previously described (Piasecka et al., 2018). To identify gene expression differences between responders and low responders, a multiple linear regression approach was performed where cell proportions from the lineage panel (leukocytes, B cells, T cells, NK cells, monocytes, neutrophils) were used to regressed out any gene expression differences due to cell population differences between donors. The analysis was implemented using the R package "broom"' v0.5.2. Multiple testing correction (Benjamini-Hochberg FDR) was then applied to select the significant genes.

Protein secretion analysis

Supernatants from whole-blood TruCulture supernatants were analyzed by Myriad RBM (Austin, Texas, US) using the Luminex xMAP technology. Samples were analyzed according to the Clinical Laboratory Improvement Amendments (CLIA) guidelines. The least detectable dose (LDD), lower limit of quantification (LLOQ) and lower assay limit (LAL) were defined as previously described [13]. To quantify interferons (α, β, γ), IL-12p70, IL-12p40 and IL-23 at ultrasensitive concentrations, Single Molecule array (Simoa) technology was used and homebrew assays developed as previously described and reported in Table S8. All the assays were run on a 2-step configuration on the Simoa HD-1 Analyzer (Quanterix, US). Limit of detection (LoD) was defined (using the highest bottom value of 95% CI in Prism 8).

Protein secretion and gene expression kinetic analysis

Time series analysis on the protein secretion and gene expression measurements, across 5 different donors, was conducted using a linear mixed-model approach. The time dependency was modelled by incorporating the parameter time as a continuous linear predictor, alongside the other protein or gene predictors, and the donors were modelled as a random effect. The model was implemented using the R package « nlme » v3.1.140.

Type I IFNβs pathway inhibition

Whole blood was diluted 1:3 with RPMI in the presence or absence of IFNβAR1 (Anifrolumab) and IFNβAR2 (PBL, clone MMHAR-2) blocking antibodies at the final concentration of 10 μg/mL. IFNα, IFNβ and IFNγ were added 1 h after the IFNβAR1 and IFNβAR2 antibodies at the final concentration of 1000 IU/mL. Samples were incubated at 37°C for 22 h and supernatants were collected for protein secretion analysis.

Intracellular cytokine staining

Fresh heparinized whole blood of healthy donors was diluted 1:3 with RPMI + GlutaMAX (Gibco, Life Technologies) without any stimulant (null) or with 10 ng/mL of LPS (InvivoGen). Diluted blood was incubated at 37°C and Brefeldin A (GolgiPlug, BD Biosciences) was added 30 min post stimulation to stain for IFNβ cytokine or 8 h after the beginning of the stimulation to stain for IL-12p70 and IL-23 cytokines. After 6h or 22h of stimulation, to detect IFNβ or IL-12p70 and IL-23 cytokines respectively, whole blood was centrifuged at 500g for 5 min at room temperature and supernatant was removed. 2mM of EDTA (Life Technologies) was added and samples were incubated at 37°C for 10 min to detach adherent cells from the tubes. Red blood cells were lysed during 10 min using Pharm Lyse reagent (BD Biosciences). Leukocytes were then washed with PBS 1X (Gibco, Life Technologies). Live dead fixable green (Thermo Fisher Scientific) was added 1:1,000 into the cell suspension and samples were incubated for 30 min at 4°C protected from light. Cells were washed once with fresh FACS buffer (PBS +2% FBS +0.2mM EDTA). FcR blocking reagent (Miltenyi) was added to the resuspended cells followed by 10 min incubation at 4°C in the dark. Surface antibody premix was distributed and cells were incubated in the dark for 30 min at 4°C. Thereafter, cells were washed with cold PBS and resuspended into fixation/permeabilization solution (Cytofix/Cytoperm kit, BD Biosciences). Samples were incubated at 4°C for 15 min protected from light. Cells were then washed with cold PBS and resuspended in 1X perm/wash buffer (Cytofix/Cytoperm kit, BD Biosciences). A second FcR blocking step was performed on resuspended cells for 10 min at 4°C in the dark prior to performing the intracellular staining. Antibodies specific to cytokines were added with 1X perm/wash buffer (100 μL total volume) and samples were incubated for 1h at 4°C in the dark. Samples were vortexed once half way through the incubation. Cells were washed once with cold PBS and resuspended in cold PBS and immediately acquired on the cytometer. Flow cytometry data were generated using LSR Fortessa (BD Biosciences) and fcs files were analyzed using FlowJo software version 10.4.2. Statistical graphs were done using Prism 8.

QUANTIFICATION AND STATISTICAL ANALYSIS

pQTL analysis

pQTL analyses were performed using the MatrixEQTL R package (Shabalin, 2012), with detection thresholds of 1e-3 for cis-pQTLs located within 1 Mb of each gene and 1e-5 for trans-pQTLs and age, sex, smoking status and CMV serology status as covariates. For annotations Ensembl gene ids ENSG00000171855, ENSG00000168811 and ENSG00000113302 were used respectively for *IFNβB1*, *IL12A* and *IL12B* genes. Protein expression data was log transformed prior to pQTL search. Bonferonni correction for multiple testing was applied to the results and pQTLs were considered significant if $p < 4.32e\text{-}6$ (FDR<0 .05) for cis-pQTLs and $p < 2.92e\text{-}9$ (FDR<0 .05) for trans-pQTLs.

Integrative modelling

Variable selection was performed using the feature selection algorithm Boruta package of R (Kursa and Rudnicki, 2010). All selected variables were tested for multicollinearity by computing the variance inflation factor using the caret package in R. IFNβ and IL-12p70 selected variables were included in a conditional inference-based approach (Hothorn et al., 2006) using ctree function of the Party package in R.