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Study of natural immune variability to dissect cytokine response pathways

Céline Possémé

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Université de Paris
Institut Pasteur
Frontière de l'innovation en recherche et éducation

Doctoral Thesis

Study of Natural Immune
Variability to Dissect
Cytokine Response Pathways

Céline Possémé

Advisor: Dr. Darragh Duffy

September 2020

In front of a jury composed of:

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Referees : Dr. Marlène Dreux (ENS Lyon)
Dr. Martin Larsen (Centre d'Immunologie et des Maladies Infectieuses)
Examinators : Dr. Lars Rogge (Institut Pasteur)
Prof. Cliona O'Farrelly (Trinity College Dublin)
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Advisor : Dr. Darragh Duffy (Institut Pasteur)

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List of Acronyms

AEB	Average enzymes per bead
AP-1	Activator protein 1
APC	Antigen presenting cell
BAI1	Brain-specific angiogenesis inhibitor 1
BDNF	Brain-derived neurotrophic factor
BCG	Bacille Calmette-Guerin
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cQTL/pQTL	Cytokine quantitative trait loci/Protein quantitative trait loci
CLIA	Clinical laboratory improvement amendments
CMV	Cytomegalovirus
CSV	Comma separated value
CTL	Cytotoxic T lymphocytes
CXCL	C-X-C motif chemokine ligand
DAA	Direct-acting antiviral agents
DC	Dendritic cell
DGE	Differential gene expression
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
eCRF	Electronic case report form
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELMO	Engulfment and cell motility
FDR	False discovery rate
FOV	Fields of view
FOXP3	Forkhead box P3
GBP	Guanylate binding protein
HCV	Hepatitis C virus
HFGP	Human functional genomic project
HIV	Human immunodeficiency virus

HLA-DR	Human Leukocyte Antigen – DR isotype
ICAReB	Clinical Investigation and Access to BioResources
IFN	Interferon
IFNAR	Interferon-Alpha/Beta receptor
Ig	Immunoglobulin
IKK	I κ B Kinase
IL	Interleukin
IL-12R	IL-12 receptor
ILCs	Innate lymphoid cells
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor 3
JAK	Janus activated kinase
LAK	Lymphokine-activated killer
LAL	Lower assay limit
LBP	LPS-binding protein
LCMV	Lymphocytic choriomeningitis
LLOQ	Lower limit of quantification
LOD	Limit of detection
LPS	Lipopolysaccharide
LRR	Leucin-rich repeats
LTBI	Latent tuberculosis infection
MAIT	Mucosal-associated invariant T cells
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial anti-viral signaling
Mb	Megabases
MD-2	Myeloid Differentiation factor 2
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Milieu Interieur
MMP-9	Matrix metalloproteinase 9
moDCs	Monocytes derived dendritic cells
MS	Multiple sclerosis
MSMD	Mendelian susceptibility to mycobacterial disease

<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor-kappa B
NGS	Next generation sequencing
NK	Natural Killer
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCR	Polymerase chain reaction
PI ₃ K	Phosphatidylinositol 3-kinase
PLC γ 2	Phospholipase C γ 2
PMNs	Polymorphonuclear leukocytes
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RCC	Reporter code count
RGP	Resorufin- β -D-galactopyranoside
RIN	RNA integrity number
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
ROR γ T	Receptor-related orphan receptor γ T
ROS	Reactive oxygen species
RT	Room temperature
SATVI	South african tuberculosis vaccine initiative
SBG	Streptavidin- β -galactosidase
SCF	Skp, Cullin, F-box
SFK	Src-family kinase
Simoa	Single molecule array
SLE	Systemic lupus erythematosus
SMOC	Supra molecular organizing center
SNP	Single nucleotide polymorphism
STAR	Spliced transcripts alignment to a reference
STAT	Signal transducers and activators of transcription

STING	Stimulator of interferon genes
SVR	Sustained virological response
TANK	TRAF family member-associated NF-kappa-B activator
TB	Tuberculosis
TBK1	TANK binding kinase 1
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIR	Toll-interleukin-1 receptor
TIRAP	Toll/interleukin-1 receptor domain-containing adapter protein
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factors
TRAM	Translocating chain-associating membrane
TRIF	TIR-domain-containing adapter-inducing interferon- β
TYK	Tyrosine kinase
UBC13	Ubiquitin-conjugating enzyme E2 13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1A
VEGF	Vascular endothelial growth factor
VST	Variance stabilizing transformation

Summary

Cytokines are essential instigators and regulators of immune responses and coordinate the response against pathogens and tumors. Therefore, they hold great potential as targets for new diagnostic and therapeutic strategies. IL-12p70 is a heterodimeric pro-inflammatory cytokine which regulates both innate and adaptive immunity and has a central role in Th1 responses. Despite its essential role in immunity, we have observed highly variable IL-12p70 responses in the 1,000 healthy donors of the *Milieu Interieur* cohort. Furthermore, this perturbed response was observed at significantly higher frequencies in patients chronically infected with tuberculosis (TB) or chronic hepatitis C (HCV). We have applied a systems biological approach in the *Milieu Interieur* cohort to dissect this perturbed pathway in healthy donors and potentially provide new insights into disease pathogenesis. Following whole blood LPS stimulation using a highly standardized approach from 1,000 healthy donors of the cohort, 28% of these donors failed to secrete IL-12p70. Having identified by flow cytometry that monocytes and dendritic cells are the main IL-12p70 producers in whole blood, we confirmed that cellular differences were not responsible for the observed variability in healthy donors. In contrast, genetic and epigenetic analysis of the healthy population cohort revealed novel associations with IL-12p70 levels. A detailed kinetic analysis allowed to define that early IFN β secretion determines variable downstream IL-12p70 responses, acting through IFNAR1 signaling. The induction of IL-12p70 by IFN β through TLR4 activation was altered in HCV infected patients who did not respond to the IFN α -based therapy. All together, these results contribute to a better understanding of IL-12p70 immune response upon bacterial infection both in health and diseases.

Résumé

Les cytokines sont des initiateurs et des régulateurs essentiels des réponses immunitaires et coordonnent la réponse contre les agents pathogènes et les tumeurs. Elles présentent donc un grand potentiel en tant que cibles pour de nouvelles stratégies diagnostiques et thérapeutiques. L'IL-12p70 est une cytokine hétérodimérique pro-inflammatoire qui régule à la fois l'immunité innée et l'immunité adaptative et joue un rôle central dans les réponses Th1. Malgré son rôle essentiel dans l'immunité, nous avons observé des réponses IL-12p70 très variables chez les 1000 donneurs sains de la cohorte *Milieu Interieur*. De plus, cette réponse perturbée a été observée à des fréquences significativement plus élevées chez les patients atteints de tuberculose (TB) ou d'hépatite C (HCV). Nous avons appliqué une approche de biologie des systèmes dans la cohorte *Milieu Interieur* pour disséquer cette voie chez les donneurs sains et potentiellement fournir de nouvelles informations sur la pathogénie de certaines maladies. Après stimulation du sang total des 1000 donneurs de la cohorte *Milieu Interieur* avec LPS, 28% des donneurs sécrètent une faible quantité d'IL-12p70. Ayant identifié par cytométrie de flux que les monocytes et les cellules dendritiques sont les principaux producteurs d'IL-12p70 dans le sang, nous avons confirmé que les différences cellulaires n'étaient pas responsables de la variabilité observée chez les donneurs sains. En revanche, l'analyse génétique et épigénétique de la cohorte a révélé de nouvelles associations avec les niveaux d'IL-12p70 sécrété sous stimulation LPS. Une analyse cinétique détaillée a permis de définir que la sécrétion d'IFN β , agissant par le biais de la voie de signalisation IFNAR1, détermine la sécrétion d'IL-12p70. L'induction de l'IL-12p70 par IFN β via l'activation du récepteur TLR4 par LPS est modifiée chez les patients infectés par HCV qui ne répondent au traitement basée sur la prise d'IFN α . L'ensemble de ces résultats contribue à une meilleure compréhension de la réponse immunitaire de l'IL-12p70 lors d'une infection bactérienne chez les individus sains et malades.

Part I

Introduction

I. Cytokines

Cytokines have key roles in cell mobility, proliferation and differentiation. They are also essential instigators and regulators of immune responses and coordinate the response against pathogens and tumors. Therefore, they hold great potential as targets for new diagnostic and therapeutic strategies. However, many questions remain regarding the intrinsic cellular and molecular mechanisms of each cytokine both in disease and healthy settings [1, 2]. The aim of my PhD thesis is to dissect immune response variability, in particular focusing on IL-12p70 cytokine responses that may determine variable responses in the context of bacterial and viral infections as well as autoimmune diseases.

I.I Classification and functions

Cytokines are small signaling proteins for cell to cell interactions and communication. They play a key role in the instigation and regulation of both the innate and adaptive immune responses. Upon infection or tumor development, cytokines guide the inflammatory responses which can drive protective immunity or be detrimental and lead to immunopathology [1].

There are 4 broad categories of cytokines:

- Interleukins; originally defined as produced by leukocytes and acting on leukocytes
- Interferons; named due to their ability to interfere with viral replication
- Chemokines; mediating chemoattraction between cells
- Tumor necrosis factors (TNF); known to inhibit tumorigenesis and induce fever

Cytokines can act in three different ways: autocrine (act on the same cell that secreted it), paracrine (act on nearby cells) and endocrine (act on distant cells). They can also have pro and anti-inflammatory pleiotropic effects, mediate similar functions and be redundant or act synergistically or antagonistically. Lastly, cytokines can mediate intracellular signaling cascades and act on cells inducing the production of other cytokines which in turn may promote further cytokine secretion [3].

1.2 Cytokine secretion variability studies

Identifying factors of cytokine variation in healthy subjects can disclose causes that lead to aberrant cytokine secretion in immune-related disorders and the ability to treat these diseases. The discovery of biomarkers for early identification and control of diseases is made possible by large-scale studies. Several research teams have investigated the impact of host and environmental factors on cytokine variation in large cohorts of healthy individuals both in baseline plasma and stimulated conditions.

1.2.1 Baseline plasma level studies

First large-scale proteome characterization using mass spectrometry in population-based cohort studies mainly focused on the impact of genetic on the plasma proteome without considering the contribution of non-heritable factors on protein levels [4, 5]. One of the first high-throughput protein quantification study performed by Johansson et al., measured 163 proteins in the plasma of 1,060 Swedish subjects. The abundance of 31 proteins was found to be influenced by genetic variants that were for the most part cis-acting SNPs [4].

The majority of recent studies integrate the impact of both genetic and nongenetic factors on protein abundance in humans. A total number of 77 biomarkers known to be involved in cancer and inflammatory diseases were measured in the plasma in a cohort of 1,005 healthy Swedish individuals [6]. This study led to the identification of 16 novel genetic associations influencing variation of biomarker levels in baseline plasma. Several non-genetic factors also have strong effects on biomarker levels with age, weight and blood pressure influencing a large number of the measured proteins. As twins provide a good opportunity to assess the relative contribution of heritable from non-heritable influences to plasma protein levels, Brodin et al., assessed the impact of genetic and non-genetic factors on 51 proteins measured in the serum of 105 healthy twin pairs. In this study, they found that non-heritable factors influence the levels of 77% of all measured parameters including cell population frequencies and serum proteins. Moreover, variations of some parameters increased with age indicating the important role of environmental exposure as a source of heterogeneity between healthy individuals [7]. Another twin cohort study investigated the heritability of protein levels in plasma and their longitudinal stability overtime. Liu et al., measured 342 proteins in the plasma of 116 healthy twins with 2 repeated sampling between 2 to 7 years after the first visit. From this study,

13 cis-acting SNPs were significantly impacting the level of specific proteins. Moreover, significant quantitative variability was observed for 17.5% of the proteins between the two timepoints [8]. Zhong et al., also performed a longitudinal study combining whole-genome sequencing, clinical phenotyping and measurement of 794 plasma proteins in 101 healthy individuals between 50 and 65 years old with 6 repeated sampling over a 2-year study period. Larger inter-individual variation was observed compared to the intra-individual variation as blood level of many proteins were stable during the study period. They found that environmental components had impact on 186 proteins and that weight loss and infection had effect on several proteins. In addition, 144 variants showed strong associations with 107 proteins leading to the conclusion that the levels of many proteins in baseline plasma of adults are determined at birth by genetics [9].

Thus, all these studies in healthy cohort mainly describe cis-acting pQTLs and show that both genetic and non-heritable factors explain protein-level variance. As the abundance of serum proteins plays an important role in the diagnosis and treatment of some diseases, the role of genetic variation in regulating protein level variation has also been investigated in several diseases such as cardiovascular diseases [10], obesity [11] or coronary disease-risk variants [12].

1.2.2 Induced immune responses studies

The human functional genomic project (HFGP) has assessed the effects of genetics, non-genetic host, and environmental factors on cytokine responses in a cohort of 489 healthy subjects after immune stimulation. These stimulations allow to mimic and reproduce infection conditions and thus, assess cytokine variability in immune responses. A total of 19 microbial and metabolic immune stimulations were performed in three different *ex vivo* cellular systems including whole-blood, peripheral blood mononuclear cells (PBMCs) and macrophage stimulations. This study highlights the effect of age, gender and seasonality on cytokine secretion. As an example, IL-22 and IFN γ secretion is reduced with age, and monocyte cytokine secretion decreases in winter [13]. In parallel, cytokine quantitative trait loci (cQTL) analysis were performed to identify genetic variants that can influence the response of 6 cytokines. This analysis revealed 17 novel genome-wide significant loci associated with cytokine secretion that were nearly all trans-QTLs. The loci of the SNPs were linked with infectious and immune-mediated diseases. This study highlights high genetic heritability of cytokine secretion and inter-individual variability in the context of microbial stimulation [14]. It has been proposed by Schirmer et

al., that non-heritable factors mainly explain inter-individual cytokine secretion variability in the baseline immune system while genetic factors predominantly explain cytokine production variability upon whole blood or PBMCs pathogen stimulation [15].

1.3 Assessing human immune responsiveness using standardized TruCulture assays

As described above, cytokine secretion varies greatly among individuals. Their production differs based on activating signals, physiological factors (such as stress and nutrition), genetics and environment [13, 14, 6]. It is essential to characterize physiological cytokine ranges to understand how cytokines interact and are modulated in healthy individuals, and to define cutoff values for potential disease diagnosis. As an example, IFN γ cytokine production upon *Mycobacteria tuberculosis* antigen whole blood stimulation is used to distinguish healthy donors from tuberculosis infected subjects [16]. However, few studies have assessed the factors that determine variance in cytokine levels in both healthy donors and disease cohorts. These research projects require to define the induced cytokine signatures in response to pathogens to capture natural occurring inter-individual diversity of immune responses. The comprehension of cytokine secretion heterogeneity can be performed using whole-blood collection stimulation assays in TruCulture tubes. This system reproduces the induced immune responses in a standardized manner. On one hand, whole blood reflects natural responses to infection as it contains all circulating immune cells including granulocytes and platelets as compared with PBMCs. On the other hand, TruCulture tubes minimize contamination and require only 1ml of whole blood. It also necessitates minimal sample handling as the blood is directly drawn into the tube. This standardized assay has been shown to be a reproducible approach to prevent technical and multi center variations [17]. This whole-blood stimulation system has been implemented to assess the inflammatory signature of the 1,000 healthy subjects of the *Milieu Interieur* cohort.

2. LabEx *Milieu Interieur* project

The *Milieu Interieur* (MI) 1,000 healthy donor cohort allows the study of naturally occurring cytokine response variability using standardized and robust immune monitoring tools. This project provides cytokine reference values from a healthy population both at mRNA transcript and protein levels. The immune system is responsible for maintaining homeostasis. Disruption of the equilibrium of the *Milieu Interieur* (as defined by Claude Bernard [18]) is associated with increased susceptibility to pathogen infections, autoimmune disease or development of cancer. Assessing the naturally occurring variability of the human immune system in healthy state is required to understand immune-mediated diseases. In other words, the evaluation of inter and intra-individual heterogeneity regarding host-related factors together with environmental factors in a healthy population will allow to define the healthy state and thus better characterize pathological states as well as predict response efficiency to therapy. This requires non-invasive, easy to perform and reproducible assay systems to assess true biological variability and generate datasets that will be comparable between healthy and disease cohorts.

The MI project is a population-based study which aims to define the boundaries of a healthy immune response. To do so, 1,000 healthy subjects of western European ancestry stratified by sex (1:1 sex ratio) and age (20 to 69 years old) were recruited [19]. To investigate the determinants of human immunologic variance, different levels of data have been acquired (Figure. 2.1):

- Environmental data were collected through a detailed medical, dietary and lifestyle questionnaire [19].
- Composition of blood cells was assessed by quantifying the immune cells and cell surface markers on fresh blood using flow cytometry [20].
- Genomic variability was measured by performing genome-wide DNA genotyping [21].
- Metagenomic diversity was determined in fecal and nasal samples through bacterial, fungi and viral sequencing [22].
- Induced transcriptional and protein inflammatory signatures by innate and adaptive

stimuli (including whole microbes, microbe-associated molecular patterns, clinically used cytokines and T cell agonists) using the standardized, easy to use whole blood TruCulture assay system. For this, whole blood was stimulated for 4 or 22 hours at 37°C in TruCulture tubes. Supernatants containing secreted proteins were collected and separated from the cell pellets which were lysed in order to extract mRNA and measure gene expression [23, 24].

The MI dataset provides reference values that reflect naturally occurring variability of the immune responses among healthy European individuals. Integration and association of these data through a systems biology approach will allow to identify genetic and environment factors that contribute to the observed diversity of immune responses.

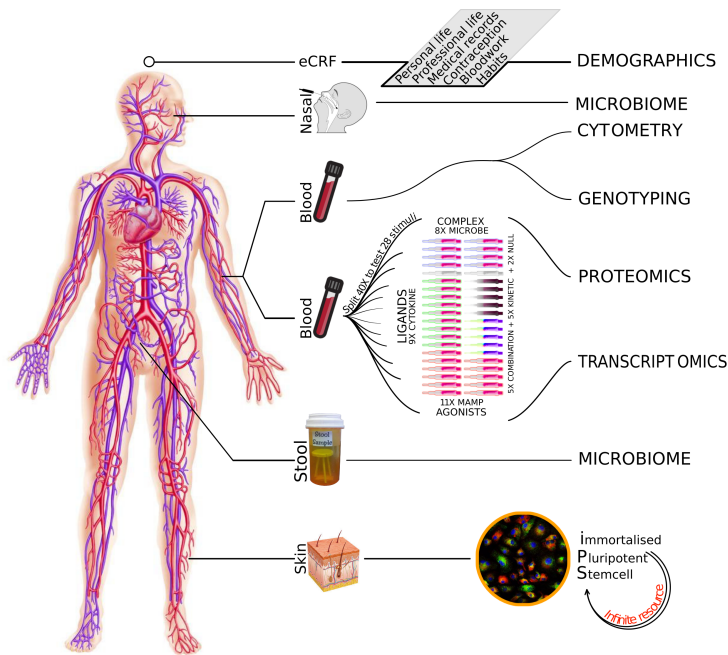


Figure 2.1: **Milieu Interieur project overview.** An extensive questionnaire regarding every-day life habits as well as nasal swabs, fresh whole-blood, stool and skin samples were collected from 1,000 healthy donors to assess the factors of human immunologic variance.

My PhD research project is based on initial analysis of this collection which revealed interesting variability regarding IL-12p70 cytokine secretion. Following whole blood lipopolysaccharide (LPS) stimulation of 25 donors of the MI cohort, 24% of these healthy donors did not secrete detectable levels of IL-12p70 [23].

3. IL-12 family of cytokines

As mentioned above, previous published observations revealed high levels of inter-individual variability in IL-12p70 cytokine responses after whole blood LPS stimulation. The IL-12 family of cytokines is part of the IL-6 superfamily and comprises four heterodimeric members: IL-12p70, IL-23, IL-27 and IL-35. This family is characterized by a unique set of connections between an α -chain (p35, p19 and p28) and a β -chain (p40 and Ebi3). The p40 subunit can bind with p35 or p19 to form IL-12p70 and IL-23 cytokines respectively, whereas Ebi3 can pair with p35 to form IL-35, or p28 to produce IL-27. IL-27 and IL-35 pair poorly and are secreted in lower amounts than IL-23 and IL-12p70 which are formed with disulfide bonds and are highly secreted. The competition dynamics for pairing of subunits and subsequent secretion remain to be defined [25].

In addition to heterodimers, some chains can be secreted without any partner. For example, p40 and p28 alone can function autonomously as monomers or homodimers. The receptor chains of this family are also composed of two chains which can be used by several cytokines. Signaling through all of these receptors is mediated by proteins of the Jak-STAT pathway (Figure. 3.1).

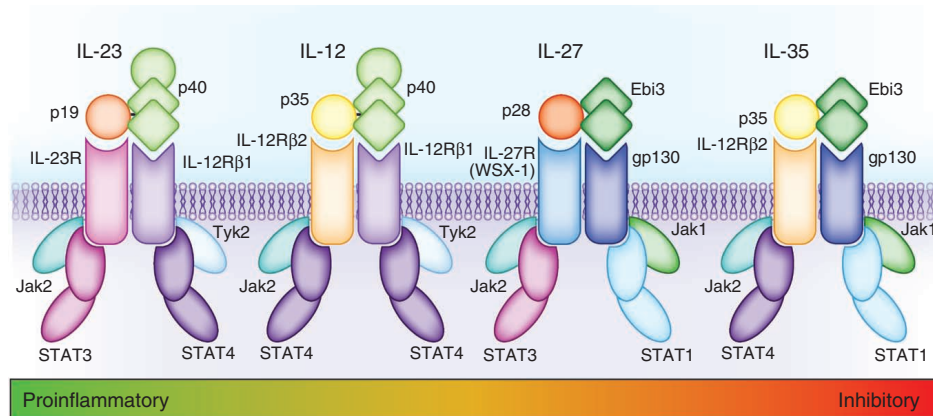


Figure 3.1: **Cytokine and receptor composition of the IL-12 cytokine family**[25]. Architecture of the four heterodimeric members of the IL-12 cytokine family together with their respective receptors and Jak-STAT signaling proteins involved.

The four IL-12 cytokines mediate diverse biological effects providing both positive and negative feedback controls. IL-12p70 is a proinflammatory cytokine which plays a

key role in the development of Th1 responses. IL-12p70 induces IFN γ production by T cells and NK cells upon antigen presentation. IL-23 is also a proinflammatory cytokine which participates in Th17 responses. IL-27 is an immunoregulatory cytokine and can have both inhibitory and prostimulatory functions. The most recently identified cytokine member, IL-35 can inhibit development of Th1 and Th17 responses and elicit the development of regulatory T cells [25].

For this project, we focused on the proinflammatory IL-12p70 and IL-23 cytokines and their shared p40 subunit that is induced upon bacterial whole blood stimulation as a model of cytokine variability. As such the rest of the introduction will focus on their regulation and function.

4. IL-12p70 cytokine

IL-12p70 is a 70 kDa heterodimeric proinflammatory cytokine that was discovered in 1989 and initially named as “natural killer cell stimulatory factor”. IL-12p70 is composed of the α -chain p35, and the β -chain p40. The two subunits are linked with disulfide bonds[26]. The two subunits IL-12R β 1 and IL-12R β 2 that compose the IL-12 receptor were fully characterized several years later[27, 28].

4.1 Biological functions

IL-12p70 regulates both innate and adaptive immunity and has a central role in Th1 responses. The main function of IL-12p70 is to exert immunoregulatory effects on NK cells and T cells. IL-12p70 induces the proliferation and cytotoxicity of NK cells as well as the differentiation and proliferation of Th1 cells characterized by expression of T-bet after antigen presentation to T cells by antigen presenting cells (APCs) [29]. Both Th1 and NK cells produce type-1 cytokines, in particular IFN γ through the activation of the Jak/STAT signaling pathway. IFN γ activates the antimicrobial functions of phagocytes and induces resistance to many bacteria, fungi and intracellular pathogens. IL-12p70 also induces production of Th1 associated immunoglobulin classes by B cells, in particular it enhances IgG1 and decreases IgE. Another action of IL-12p70 is to enhance the production of cytotoxic T lymphocytes (CTLs) and lymphokine-activated killer cells (LAK). It increases the cytotoxic activity of CTLs and NK cells by inducing gene transcription of cytotoxic granule-associated molecules including perforin and granzymes, but also by upregulating gene expression of adhesion molecules. IL-12p70 induces the up-regulation of gene expression encoding for several cytokines, chemokines, receptors, membrane molecules, signaling and effector molecules as well as transcription and nuclear factors (detailed table listing the genes upregulated by IL-12p70 described by Watford et al.[30]). Finally, IL-12p70 and IFN γ antagonize Th2 differentiation and the production of IL-4, IL-5 and IL-13 cytokines (Figure. 4.1) [31].

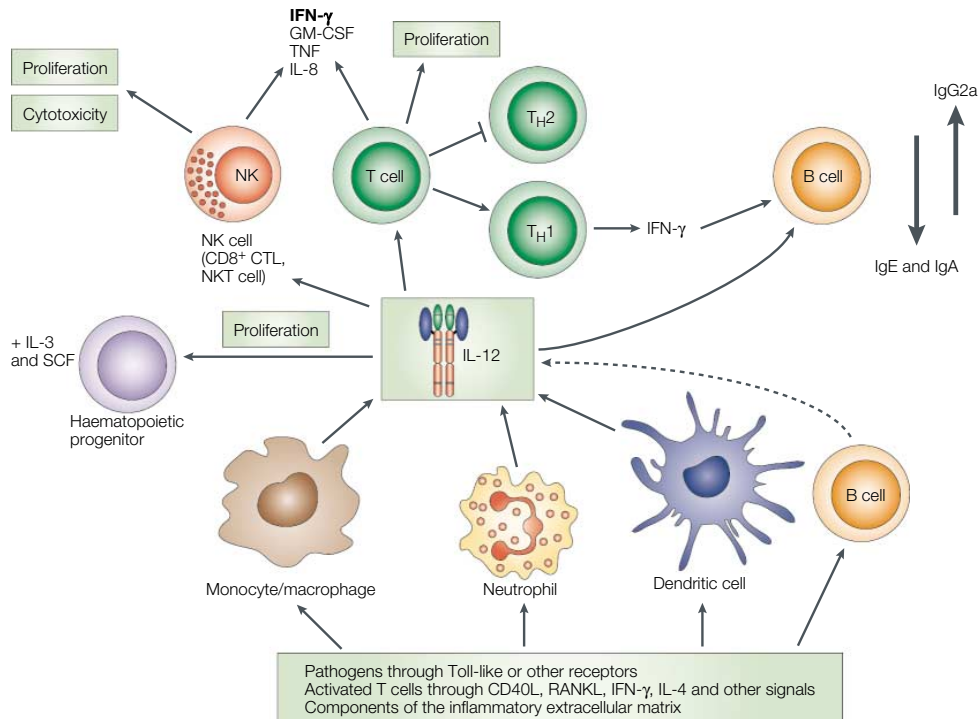


Figure 4.1: **IL-12p70 biological functions** [31]. Presentation of the main IL-12p70 cytokine producers in response to pathogens as well as the target cells of IL-12p70 and their biological activities.

4.2 IL-12p70 production and signaling pathway

4.2.1 Encoding genes

The genes encoding the two subunits are located at two independent loci. The p35 and p40 subunits are encoded by the *IL12A* gene on chromosome 3 and the *IL12B* gene on chromosome 5 respectively [32]. Both genes have to be coordinately expressed by the same cells in order to secrete the biologically active IL-12p70 cytokine [33]. The *IL12A* gene is expressed in many cell types, including non-immune cells. By contrast, *IL12B* gene expression is restricted to cells producing the heterodimer. In the absence of p35 or p19 subunit (which binds with p40 to form IL-23 cytokine), p40 is secreted as a monomer or homodimer whereas p35 can be secreted only if associated with p40 [34].

4.2.2 IL-12p70 biosynthesis

As previously mentioned, co-expression of the two subunits within the same cell is necessary to form the biologically active IL-12p70 cytokine. The two subunits interact to

gether and are linked with disulfide bounds in the endoplasmic reticulum. Thereafter, glycosylation of the cytokine takes place in the Golgi apparatus and is essential for mature IL-12p70 cytokine secretion [35]. The p40 subunit enhances post translational stability, and promotes the intracellular trafficking and export of the p35 chain which results in secretion of IL-12p70. The absence of p40 does not allow the transfer of the p35 subunit into the Golgi complex. Moreover, the relative quantity of the two cytokines is essential in determining the efficiency of IL-12p70 secretion and excess production of the p40 chain is critical to direct the transport of p35 and thus maximize the secretion of IL-12p70. The production of IL-12p70 is rapid and its rate is dictated by the availability of the p40 subunit [34].

4.2.3 Cell types secreting IL-12p70

IL-12p70 is primarily produced by antigen-presenting cells. Purified monocytes, macrophages and dendritic cells (DCs) have been shown to produce IL-12p70 in response to bacteria, intracellular parasites, viruses, fungi, dsRNA and CpG through toll-like receptor (TLR) signaling [36, 37, 38]. Using Northern blot, one study has shown that purified polymorphonuclear leukocytes can produce IL-12p70 upon LPS stimulation [39]. Human tonsillar B cells have also been reported to secrete bioactive IL-12p70 cytokine using enzyme-linked immunosorbent assay (ELISA) and intracellular flow cytometry [40]. Of important note, none of these studies were performed using human whole blood stimulation. In addition, the majority of these findings were achieved before the discovery of the IL-23 cytokine which shares the p40 subunit with IL-12p70. As a consequence, the tools used to detect IL-12p70 did not consider this parameter and detection of IL-12p70 may have been compounded by co-detection of IL-23.

4.2.4 IL-12p70 receptor and signaling pathway

The heterodimer molecule signals through the IL-12 receptor (IL-12R) comprising IL-12R β 1 and IL-12R β 2 subunits that are expressed on T cells, NK cells, DCs and some B-cell lines. IL-12R is not present on most resting T-cells but is expressed at low levels by NK cells allowing them to respond rapidly to IL-12p70. Activation of T cells via the T cell receptor (TCR) upregulates gene expression of the two IL-12R chains [41, 42, 43, 27]. Co-expression of the two transmembrane glycoprotein subunits is required for the formation of high affinity IL-12p70 binding sites. Signal transduction through IL-12R induces

tyrosine phosphorylation of the janus activated kinase 2 (JAK2) and the tyrosine kinase 2 (TYK2) which in turn mainly phosphorylate the activator of transcription, STAT4 leading to STAT4 homodimerization and translocation of the homodimer to the nucleus to promote gene transcription including IFN γ (Figure. 4.2) [44]. In addition, the PI $_3$ K/Akt pathway has been reported to be activated by IL-12p70 in mice leading to proliferation and survival of CD4 $^+$ T cells, but not IFN γ production [45].

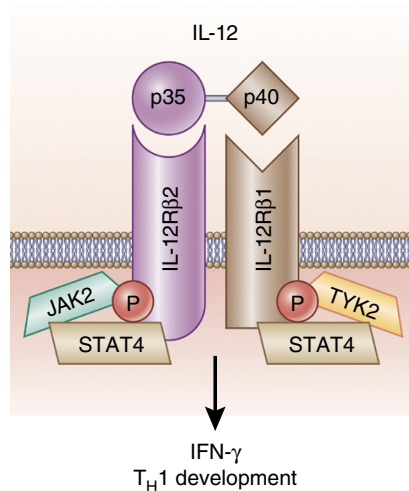


Figure 4.2: **Schematic representation of IL-12p70, its receptor and downstream signaling pathway** [46]. IL-12p70 is composed of the p35 and p40 subunits and signals through the IL-12 receptor made of the IL-12R β 1 and the IL-12R β 2 chains. IL-12p70 activates Jak2 and TYK2 molecules leading to the phosphorylation of STAT4 and the production of IFN γ triggering a Th1 immune response.

4.3 Regulation

Similar to other cytokines, the existence of numerous mechanisms controlling IL-12p70 production enables efficient and appropriate immune responses against pathogens. The positive and negative regulation of IL-12p70 activity can occur at different levels by regulatory factors. The first one corresponds to *IL12A* and *IL12B* gene expression and translation as described previously. Further regulation occurs in the IL-12p70 signaling pathway and the genes upregulated by the heterodimer. Furthermore, cells expressing multiple subunits of the IL-12 cytokine family may lead to competition for pairing and secretion. The following sections report the main positive and negative regulatory factors of IL-12p70 production.

4.3.1 Positive regulation

Bacteria, bacterial DNA, fungi, intracellular parasites, double-stranded RNA such as Poly(I:C), single-stranded RNA and CpG oligodeoxynucleotides highly activate IL-12p70 production [47, 48]. Several cytokines can also increase the capacity of cells to secrete IL-12p70. As an example, IL-12p70 positively regulates its own production *via* IFN γ , which primes monocytes and neutrophils to activate interferon regulatory factors inducing the transcription of both *IL12A* and *IL12B* genes. The ability of IFN γ to enhance production of IL-12p70 creates a positive feedback mechanism during inflammation and Th1 responses [49, 50]. IL-12p70 can also bind to its receptor on DCs to increase its own production. In addition, IL-4 and IL-13 priming before infection can also enhance IL-12p70 production in a time-dependent manner [51, 52]. In response to TLR activation, IL-12p70 secretion is also dependent on type I IFNs which regulate IL-12p35 mRNA transcription [53]. Finally, IL-12p70 production can also be facilitated through direct cell to cell interactions. In the context of bacterial infection, DCs express CD40 costimulatory markers that interact with CD40L activation markers on T cells to optimize IL-12p70 production [54].

4.3.2 Negative regulation

The anti-inflammatory cytokine IL-10 is a well-known strong inhibitor of IL-12p70 secretion by blocking the transcription of both *IL12A* and *IL12B* genes. Transforming growth factor β (TGF- β) also inhibits IL-12p70 production by reducing the stability of the *IL12B* gene [55, 56]. Moreover, signaling through many G protein-coupled receptors including chemokines CCL2-7-8-13 and complement component C5a also inhibits IL-12p70 secretion by macrophages and downstream Th1 responses [57]. Finally, prostaglandin E2 mediates the inhibition of IL-12p70 production through the inhibition of *IL12B* transcription [58].

4.4 IL-12p70 roles in diseases

Patients with genetically impaired IL-12p70/IFN γ axis immunity, also called mendelian susceptibility to mycobacterial disease (MSMD) are more susceptible to weakly virulent Mycobacteria as well as Salmonella infections [59]. Interestingly, these IL-12p70 deficient patients are protected against a secondary mycobacterial infection. Moreover,

these patients are resistant to other pathogens such as cytomegalovirus (CMV) or herpes virus showing that it is probably most unlikely that IL-12p70 is a key cytokine in the immune response of these infections [60]. IL-12p70 has been shown to be protective in some but not all viral infections [61]. As an example, IL-12p70 contributes in controlling influenza virus by enhancing cytotoxic T lymphocytes [62]. As previously mentioned, the IL-12p70 cytokine plays a key role in mounting protective immunity against bacterial infection by triggering a Th1 immune response. High levels of IL-12p70 have been reported in several auto-immune diseases such as Crohn's disease, osteoarthritis, rheumatoid arthritis, seronegative spondylarthropathy, and atherosclerosis. In addition to immune-mediated inflammatory diseases, increased IL-12p70 plasma levels are also detected in patients with neurological disorders such as multiple sclerosis (MS). Currently, several antagonist agents targeting IL-12p70 or IL-23 are being tested or have been approved for immune-mediated diseases [46]. As an example, Ustekinumab which is a monoclonal antibody targeting the p40 subunit of IL-12p70 and IL-23 blocking its interaction with the IL-12R β 1 receptor chain, has been approved for the treatment of psoriasis and Crohn's disease.

5. IL-12p40 and IL-12p80 cytokines

Both p35 and p19 subunits require IL-12p40 to be secreted. However, IL-12p40 can be produced in a monomeric or homodimeric form linked by disulfide bonds called IL-12p80 or IL-12(p40)² [63]. IL-12p40 is secreted at a 10 to 20 fold excess as compared with IL-12p70 by stimulated human blood cells. Both IL-12p40 and IL-12p80 bind to IL-12R β 1 receptor subunit and can have both antagonist and agonist effects. Few studies of IL-12p40 and IL-12p80 have been performed using human samples and, even though IL-12p80 has been detected in human samples, less is known about the availability of the homodimeric cytokine in humans. Thus, the majority of results described in this section have been performed using mouse models.

5.1 IL-12p40

The main biological function of IL-12p40 is to competitively bind to the IL-12p70 receptor creating a negative feedback loop [64]. However, it has been suggested that IL-12p40 is not only a negative regulator of the Th1 response but might also have an agonist role and initiate an immune response under certain conditions.

5.1.1 Biological activities of IL-12p40

In a rat tumor model system, IL-12p40 was able to amplify the immune response against tumors through the recruitment of macrophages [65]. In addition, IL-12p40 has been shown to be important for the initiation of T cell responses. Mice lacking IL-12p40 showed a defect in DC migration from the lung to the draining lymph node after *Mycobacterium tuberculosis* exposure as well as a diminished activation of naïve T cells. This defect could be overcome with the addition of exogenous IL-12p80 suggesting potential redundancy between IL-12p40 and IL-12p80 biological functions [66]. In summary, several studies highlight that IL-12p40 may have different physiological functions and act as both an antagonist of IL-12p70 and as an effector molecule.

5.2 IL-12p80

In mice, 20% to 40% of IL-12p40 in the serum is in the form of IL-12p80. In human *in vitro* studies, IL-12p80 was at least 20 times more effective than IL-12p40 at inhibiting the IL-12p70 Th1 response and at binding to the IL-12p70 receptor. However, the affinity of IL-12p80 for the IL-12 receptor is 10 to 20% of the affinity of IL-12p70 [67]. Similar to IL-12p40 functions, IL-12p80 can also act as an antagonist by binding to the IL-12p70 receptor [63, 68]. However, IL-12p80 can also trigger some biological activities.

5.2.1 Biological functions of IL-12p80

IL-12p80 can modulate myeloid cell activity and trigger TNF α and nitric oxide synthase production via IL-12R β 1. In naïve mouse splenocytes, IL-12p80 production suppressed FOXP3 and CD25 expression by regulatory T cells in a dose dependent manner [69, 70, 71]. In addition, IL-12p80 has been shown to act as a macrophage chemoattractant in both rat and mouse models [72]. This function has been shown to be detrimental in the context of allograft [73]. Moreover, IL-12p80 can promote IFN γ production by CD8 T cells participating in allograft rejection [74].

Interestingly, the functions of IL-12p80 have been investigated in the context of autoimmune arthritis and leishmaniasis in mice, and have shown different effects. In a mouse model of cutaneous leishmaniasis, local injection of IL-12p80 enhanced disease susceptibility and increased lesion volumes. Immune responses against *Leshmania* require IFN γ production and antagonist functions of IL-12p80 might be responsible for inhibiting Th1 responses in this disease [75]. However, in animal models of autoimmune arthritis, IL-12p80 suppressed inflammatory arthritis by inhibiting the production of inflammatory cytokines and T cell proliferation. Unlike the study by Brahmachari et al., IL-12p80 induced regulatory T cells whereas the generation of Th17 cells was suppressed [76]. Similar results were observed in an autoimmune colitis model in which IL-12p80 suppressed Th17 cells [77]. Thus, IL-12p80 could prevent IL-12p70 and IL-23-mediated immune diseases in different contexts by competitively binding to the IL-12R β 1 subunit of the receptor.

6. IL-23 cytokine

The IL-23 cytokine was discovered in 2000 by Oppmann et al., and its receptor was identified two years later. IL-23 is a proinflammatory cytokine composed of the p19 and p40 subunits. Like IL-12p70, the heterodimers of p40 and p19 are formed with disulfide bonds [78, 79].

6.1 Biological functions

IL-23 plays a major role in the differentiation of naïve T cells into Th17 cells. However, IL-23 alone cannot induce this differentiation. IL-6 and TGF- β are also required as inducing factors for Th17 cell differentiation, while IL-1 β can enhance the proliferation of Th17 cells. IL-23 is necessary for the maturation of Th17 cells and promotes their pathogenicity through four main mechanisms which are (i) supporting Th17 signature gene expression, (ii) inducing effector gene expression, (iii) downregulating repressive factors and (iv) amplifying its signaling pathway through the upregulation of IL-23 receptor gene expression. Th17 cells secrete several cytokines including IL-17A, IL-17F, IL-22, IL-26, CCL20 and the chemokine receptor CCR6. Both IL-17A and IL-17F are important for recruitment of neutrophils to epithelial cells. In addition, IL-17A with IL-22 induces the production of anti-microbial peptides from numerous epithelial cells [80, 81, 82]. Like IL-12p70, IL-23 also induces IFN γ expression in human Th17 cells. Nevertheless, in contrast to IL-12p70, IL-23 acts on memory T cells to promote their proliferation. Firstly, it promotes IFN γ secretion in both CD4⁺ and CD8⁺ naïve and memory T cells and secondly, it induces IL-17 secreting memory T cells. The proinflammatory cytokine IL-17A stimulates IL-1, IL-6, Tumor necrosis factor α (TNF α), nitric oxide synthase 2 (NOS-2) and inflammatory chemokines. Thus, as memory T cells highly proliferate during disease, the role of IL-23 is particularly relevant for immunological responses during bacterial and viral infections and also chronic inflammation [83, 84].

6.2 IL-23 production and signaling pathway

Activated monocytes, macrophages, DCs, keratinocytes and endothelial cells can secrete IL-23. IL-23 production is stimulated by both Gram+ and Gram- bacteria and by the activation of TLR2-8 receptors [37, 85, 86, 87]. Analogously to IL-12p70, biologically active IL-23 requires the production of both chains within the same cell and the p19 subunit needs co-expression of p40 for secretion. The *IL23A* gene is located on chromosome 12. An important epigenetic mechanism regulating both IL-12p70 and IL-23 gene expression in DCs has been identified. The deubiquitinase trabid protein has been shown to be essential to induce histone modifications at the *IL12A*, *IL12B* and *IL23A* gene promoters, which is necessary for gene transcription and thus inflammatory responses [88]. The IL-23 receptor is composed of IL-23R and IL-12R β 1, also shared with the IL-12p70 receptor. IL-23R associates with JAK2 while IL-12R β 1 interacts with TYK2. IL-23 binding leads to phosphorylation and activation of JAK-STAT signaling molecules. STAT3 or STAT4 are phosphorylated and form homodimers that shuttle into the nucleus and bind to STAT binding sites in the promoter. STAT4 is necessary to induce IFN γ gene transcription whereas STAT3 phosphorylation is essential for Th17 cell development and induces the expression of IL-17 and ROR γ T genes (Figure. 6.1). IL-23 activates less STAT4 than IL-12p70, and STAT3 is the predominant activated STAT upon IL-23 cytokine signaling [30].

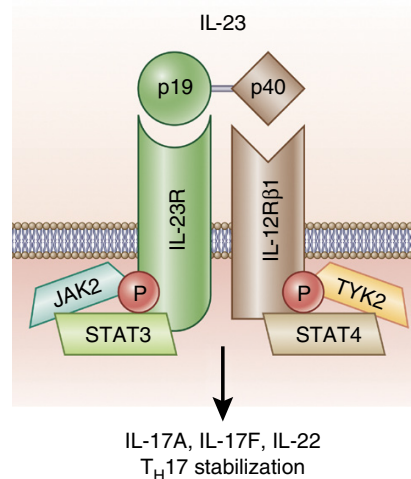


Figure 6.1: **Schematic representation of IL-23, its receptor and downstream signaling pathway [46].** IL-23 is made up of the p35 and p40 subunits and signals through the IL-23 receptor comprised of the IL-12R β 1 and the IL-23R chains. IL-23 activates JAK2 and TYK2 molecules leading to the phosphorylation of mostly STAT3 and the production of IL-17A, IL-17F and IL-22 cytokines inducing a Th17 immune response.

6.3 IL-23 cytokine in disease

IL-23 imbalance can lead to autoimmune inflammatory disorders and pathogenesis. IL-23 has been extensively used as a clinical indicator of immune-mediated diseases. Deregulated levels of IL-23 mainly affects barrier functions and leads to pathologies of skin, gut and lung. Patients with psoriasis autoimmune disease present high levels of IL-23 in their lesions [89]. A new monoclonal antibody that selectively target the p19 subunit named Guselkumab has been recently approved for the treatment of moderate to severe plaque psoriasis [90]. Additionally, ankylosing spondylitis and inflammatory bowel disease such as Crohn's diseases have been genetically associated with the IL-23 pathway [46]. Targeting the cytokine itself, its receptor or signal transduction are potential therapeutic approaches for these diseases.

7. Type I interferons

A few studies have highlighted a link between type I IFNs and IL-12p70 production. Gautier et al., demonstrated that IL-12p70 production in response to TLR activation was dependent on production of endogenous type I interferons (type I IFNs) that regulated IL-12p35 mRNA accumulation [53]. Moreover, Nagai et al., observed that IFN β can trigger or inhibit Th1 responses driven by IL-12p70 depending on the time of exposure [91]. Thus, in addition to assess IL-12p70 secretion variability, we also investigated inter-individual heterogeneity of type I IFNs and the potential role of type I IFNs in variable IL-12p70 production.

7.1 Type I interferon subtypes

The term “interferon” (IFN) was defined for the first time in 1957 and described as a substance produced by cells and which interferes with influenza infection [92]. In 1978, two decades after the initial discovery of this cytokine, interferon was purified and its properties were characterized [93]. This state of progress led to the identification of a family composed of distinct proteins rather than one single IFN molecule. In humans, the type I IFN family includes several members and is composed of 13 type I alpha IFNs (*IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA10*, *IFNA13*, *IFNA14*, *IFNA16*, *IFNA17* and *IFNA21* genes), one IFN β (*IFNB1* gene) as well as IFN ϵ (*IFNE* gene), IFN κ (*IFNK* gene) and IFN ω (*IFNW1* gene). All genes are clustered on the short arm of chromosome 9. DNA sequences show that the human *IFNA* gene family shares 70 to 80% sequence homology within the 13 subtypes and 35% identity with the *IFNB1* sequence [94, 95].

7.2 Type I interferon production

Secretion of type I IFN is induced via stimulation of pattern recognition receptors (PRRs) that detects bacterial and viral nucleic acids or compounds and endogenous ligands. Stimulated PRRs induce signaling pathways that activate transcription factors that are members of the interferon regulatory factor (IRF) family which in turn triggers the

production of type I IFNs [96, 97]. In the context of LPS stimulation, IFN β was shown to be secreted by bone marrow derived macrophages as well as bone marrow derived DCs using mouse cell lines [98]. In humans, primary purified monocytes expressed IFN β mRNA 1 hour after the beginning of the stimulation demonstrating the quick IFN β induction by monocytes [99]. The secretion of IFN α by pDCs after exposure to virus has been well characterized [100, 101]. However, TLR $_4$ activation by LPS also induces IFN α production [24]. It has been demonstrated that mouse cDCs as well as human purified monocytes secrete IFN α upon LPS stimulation after IFN β priming, showing that these two cell types are able to produce IFN α upon TLR $_4$ activation [102].

7.3 Type I interferon signaling pathway

Multiple signaling pathways are activated by type I IFNs and the cooperative functions of these signaling cascades are important for the induction of biological responses.

7.3.1 Activation of classical JAK-STAT pathways

Both IFN α and IFN β bind to the same heterodimeric transmembrane receptor IFNAR composed of two subunits: IFNAR $_1$ and IFNAR $_2$ which are associated with TYK $_2$ and JAK $_1$, respectively. IFNAR $_2$ binds interferon molecules with high affinity while the IFNAR $_1$ subunit binds the proteins with low affinity. IFN β presents the highest binding affinity among all type I IFNs [103]. In the canonical type I IFN signaling pathway, IFNAR engagement leads to dimerization of the IFNAR subunits followed by the autophosphorylation and activation of JAK $_1$ and TYK $_2$. The latent cytoplasmic signal transducers and activators of transcription STAT $_1$ and STAT $_2$ are tyrosine-phosphorylated and dimerize [104, 105, 106]. This heterodimer assembles with IRF9 to form the IFN-stimulated gene factor 3 (ISGF3) complex [107, 108, 109]. ISGF3 translocates to the nucleus and binds to IFN-stimulated response element (ISRE) sequences to induce expression of several hundred genes. The majority of these interferon stimulated genes (ISGs) are classical antiviral genes [110, 111] (Figure. 7.1). In addition to the canonical pathway, IFNAR activates STAT $_1$ and STAT $_3$ homodimers to a minor extent in the majority of cell types. STAT $_1$ homodimers bind to gamma-activated sequences (GASs) to induce the expression of pro-inflammatory genes [112, 113] whereas STAT $_3$ homodimers indirectly induce genes that suppress inflammatory responses through its association with the co-repressor complex SIN3A [114]. IFNAR can also activate STAT $_4$,

STAT5 and STAT6 in some cell types in a context-dependent manner. These other STAT molecules form homodimer or heterodimer complexes or also bind with co-activators (for example STAT5 can associate with CRKL protein). These complexes translocate to the nucleus and also bind GAS elements [115].

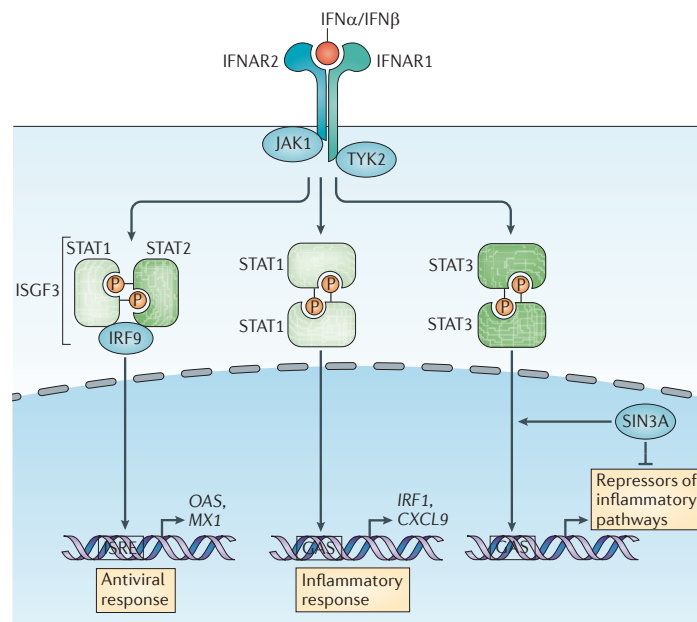


Figure 7.1: **Canonical type I interferon JAK-STAT signaling pathways [115]**. Activation of the type I IFN receptor composed of IFNAR₁ and IFNAR₂ subunits leads to the activation of Jak1 and Tyk2 molecules. These two proteins induce the activation, dimerization and nucleus translocation of STAT proteins which in turn activate the transcription of genes involved in antiviral and inflammatory responses.

7.3.2 Activation of independent JAK-STAT pathways

The p38-signaling pathway has been shown to be activated by type I IFNs in cell lines. Type I IFNs can activate mitogen-activated protein kinases (MAPK) that phosphorylate p38 and several effector kinases such as MSK₁ or MAPKAPK₂ are in turn activated [116, 117]. These effector kinases have regulatory effects on gene transcription. This signaling cascade has growth-inhibitory effects and is essential for the induction of antiviral responses in patients infected by the hepatitis C virus (HCV) highlighting the functional relevance of this pathway [118, 119]. In addition, type I IFNs can activate the PI₃K-signaling pathway which results in downstream formation of STAT₁ homodimers which bind GAS elements as previously described [120]. Interestingly, it has also been demonstrated in mice that IFN β can bind to the IFNAR₁ subunit independently of IFNAR₂.

IFN β -IFNAR₁ association mediates specific and unconventional intracellular signals independently of Jak-STAT pathways and induces the expression of a distinct group of genes independently of the canonical type I IFN pathway [121, 122].

7.4 Regulation

Type I IFNs play critical roles in host protection from pathogens. Dysregulation of type I IFN production or responses can mediate immune pathogenesis such as autoimmune diseases (named interferonopathies) and infectious diseases through aberrant inflammatory responses or improper suppression of pathogens [123]. Thus, type I IFNs needs to be tightly regulated to achieve both protective immunity and avoid toxicity of excessive type I IFN responses.

7.4.1 Positive regulation

Production of type I IFNs is upregulated by a positive feedback loop. Type I IFNs induce IRF7 production through ISGF3 transcription factor activity. Then, IRF7 undergoes nuclear translocation and in turn induces type I IFN secretion [124]. In addition, type I IFNs induce STING (stimulator of interferon genes) expression via a STAT1 binding site and the induction of STING leads to further interferon production. Also, IRF7-dependent IFN α induction further amplifies STING expression [125].

7.4.2 Negative regulation

Elevated or persistent type I IFN responses are detrimental, causing increased levels of inflammation. Therefore, multiple layers of feedback mechanisms on the IFNAR receptor and its mediated pathways exist. At the cytokine expression level, IRF2 and p50 homodimers are negative regulators of IFN β expression. Both are constitutively expressed and attach to the IFN β promoter to act as repressors [126]. The inhibition of the type I IFN pathway is mediated through protein-protein interaction, post-translational modifications as well as post-transcriptional regulation by non-coding RNA. The list of negative regulators, their targets and the regulatory mechanism applied are reported in Table 1 (Table. 7.1).

Negative regulator	Target	Mechanism
DCST1	STAT2	Ubiquitination
IRF2	IRFs	Binding
PIAS1	STAT1	Binding
PIASy	STAT1	Inhibition of STAT1-dependent transcription
PKD2	IFNAR1	Ubiquitination
PTP1B	TYK2	Dephosphorylation
RNF2	STAT1	Ubiquitination
S1PR1	IFNAR1	Lysosomal degradation
SHP1	JAK1, STAT1	Dephosphorylation
SHP2	JAK1, STAT1, STAT2	Dephosphorylation
SMAD2, SMAD3	STAT1	Binding
SMURF1	STAT1	Ubiquitination
SOCS1	TYK2	Binding
SOCS3	JAK1	Binding
SPRY1, SPRY2, SPRY4	ERK1/2	Dephosphorylation
TC-PTP	JAK1	Dephosphorylation
USP18	IFNAR2	Binding

Table 7.1: **Negative regulators of Type I IFN response together with their target proteins and mechanism of action [123].**

7.5 Roles in disease - Biological functions

Type I IFNs are pleiotropic cytokines with beneficial or detrimental effects in infection and cancer. These cytokines are also essential to link the innate and adaptive immune systems. Dysregulation of the type I IFN response leads to a breakdown of immune tolerance and autoimmune diseases.

7.5.1 Viral infections

The protective role of type I IFNs in early stages of viral infection and in the resolution of acute infections has been well characterized [127]. Upon pathogen-encounter, type I IFNs are produced and trigger the expression of a diverse set of ISGs inducing a potent antiviral response. Some ISGs are involved in antiviral activities while others specifically target different classes of viruses. ISG-encoded proteins are composed of direct effectors mediating the antiviral response through several mechanisms (inhibition of viral replication, transcription and translation as well as degradation of viral genome and alteration of cellular lipid metabolism) and factors which induce adaptive immune responses. Nevertheless, the effects of prolonged type I IFN secretion are not well characterized in chronic viral infections. In persistent infections such as HIV (human immun-

odeficiency virus), elevated viral loads correlate with the level of type I IFNs and ISG expression [128]. Whether prolonged type I IFN production leads to viral persistence or chronic infection still needs to be investigated [129].

7.5.2 Bacterial infections

Unlike viral infections, the role of type I IFNs in bacterial infections is less clear. They can be protective or detrimental during bacterial infection depending on the pathogen and the stage of infection. As type I IFNs induce genes that modulate both the innate and adaptive immune response, these genes can be beneficial for the formation of antibacterial inflammatory responses while others can be detrimental [130]. As an example, type I IFNs exacerbate bacterial infection during *Listeria monocytogenes* or *Staphylococcus aureus* infections. Different mechanisms of action have been highlighted, among them type I IFNs can attenuate the expression of the IFN γ receptor and they can also inhibit the expression of IL-17A by $\gamma\delta$ T cells which suppress neutrophil recruitment. In contrast, type I IFNs are protective during *Streptococcus pneumoniae* or *Helicobacter pylori* infections through the induction of ISGs such as CXCL10. The impact of type I IFNs on the outcome of bacterial infections is explained by different factors including virulence factors, the bacterial replication strategy and the site of the infection [113].

7.5.3 Autoimmune diseases

As previously described, production of type I IFNs upon pathogen infection activates a positive feed-forward self-amplifying loop to ensure rapid and efficient virus eradication. However, this auto-amplification loop can be involved in the pathogenesis of certain autoimmune diseases due to aberrant activation of immune responses. First, autoimmunity can be induced by type I IFN based immunotherapy [131]. This treatment is used to treat chronic viral infections such as hepatitis C and B. However, this therapy has been associated with the development of autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) in some patients [132]. As this phenomenon is rare and reversible, it is suggested that additional genetic and environmental factors play a role to induce this autoimmunity associated with type I IFN based therapy. Second, many studies have shown increased expression of ISGs in patients with autoimmune disorders such as SLE and interferonopathies [133, 134, 135]. For the majority of patients, the expression was correlated with disease severity. Third, host genetic poly-

morphisms in type I IFN pathways have been identified and linked with an increased risk to several autoimmune diseases. As an example, genetic polymorphism in STAT4 protein, activated downstream type I IFNs pathway, have been linked with susceptibility to RA and SLE diseases [136]. Finally, in patients with SLE, it has been reported that monocytes can differentiate into DCs in an IFN α -dependent manner. These matured myeloid DCs activate in turn autoreactive T cells. pDCs and autoreactive T cells help expand autoreactive B cells leading to the destruction of host tissues through the production of autoantibodies [137]. All these findings highlight the potential of type I IFNs for amplifying immunopathology in autoimmunity [138, 139].

7.6 Differences between IFN α vs IFN β

Many published studies present results and conclusions about type I IFNs without differentiating between IFN α and IFN β . However, although they signal through the same receptor and have functional overlaps, IFN α and IFN β present distinct roles, and it is likely that they are evolutionarily preserved for selective functions. Also, IFN α and IFN β are clinically used to treat different diseases. IFN α is used to treat hepatitis B and C while IFN β treatment is used to prevent relapses in multiple sclerosis, albeit with variable success rates. The currently accepted view is that the different activities between IFN α and IFN β are related to the recognition chemistry and binding to the IFNAR receptor. The separate roles of IFN α and IFN β are supported by different studies summarized in this section.

7.6.1 Unique role of IFN α

IFN α and IFN β are both induced early after infections but they play different roles in controlling viral infections. As an example, in the case of LCMV (lymphocytic choriomeningitis) infection, IFN α is effective in managing viral spread whereas IFN β is not important for early anti-viral activity [140]. This provides a potential explanation for the effectiveness of IFN α therapy in decreasing HCV RNA. Moreover, IFN α and IFN β are not produced in the same proportion by the different cell types. As an example, upon TLR7 and TLR9 activation, pDCs produce 56 times more IFN α than IFN β (unpublished data).

7.6.2 Unique roles of IFN β

IFN β is implicated in a multitude of biological effects that have not been described for IFN α . First of all, healthy organisms present constitutive IFN β production in the absence of infection. This constitutive secretion is essential to maintain appropriate antiviral and antitumor responses as well as hematopoietic stem cell homeostasis. This has not been demonstrated for IFN α , showing that IFN β is a master regulator of type I IFNs activities [141]. The timing of IFN β secretion has been shown to be crucial in determining whether its response is beneficial or detrimental. During maturation of DCs, IFN β increases the capacity of DCs to promote Th1 cells which secrete IFN γ . However, in the presence of mature DCs stimulating naïve T cells, IFN β inhibits Th1 cell polarization and promotes the secretion of IL-10 by T cells [91]. This activity is clinically relevant in MS disease in which increased production of IFN γ precedes clinical attacks, and Th1 as well as Th17 cell migration leads to the formation of MS lesions from the periphery to the central nervous system. Thus, IFN β therapy is used in MS disease for its immunosuppressive effect that reduces the T cell responses responsible for the progression of this disease [142].

Interestingly, IFN β contains an NGR sequence motif (Asn-Gly-Arg) placed in the region involved in the IFNAR₂ binding. The NGR motif can be deaminated into DGR and iso-DGR which can then bind to integrins ($\alpha v \beta 3$ and potentially others) [143]. Although further investigations are required, this combination could be associated with the described anti-cancer immune effect of IFN β . In cancer, IFN β but not IFN α can inhibit melanoma cell migration and in triple-negative breast cancer, high levels of IFN β correlates with improved recurrence-free survival [144, 145].

7.6.3 Potential explanations for the different biological outcomes between IFN α and IFN β

The implications of IFN α or IFN β in a specific infection can be explained by the selective production of one of the cytokines in these circumstances. For example, LPS toxicity specifically triggers IFN β secretion. Factors such as timing, signaling intensity and cell source of the type I IFN subtypes play an essential role in coordinating the outcome of type I IFN signaling. It has also been shown that the relative affinity of IFN α and IFN β for the two IFNAR subunits and the resulting stability of the tertiary interferon-receptor complex induce different biological outcomes [146, 103].

8. LPS - TLR₄ signaling pathways

To study IL-12p70 and type I IFNs inter-individual secretion variability, we used Tru-Culture tubes pre-loaded with cell culture media and the LPS as an immune stimulant.

8.1 LPS

8.1.1 Composition

The surface of gram-negative bacteria is composed of numerous ligands that are recognized by TLRs of immune cells. One such ligand, LPS is a structural component of the outer membrane of gram-negative bacteria which contains two membranes in contrast to gram-positive bacteria. LPS is composed of three parts: the distal polysaccharide O-antigen, the core oligosaccharide part and lipid A (or endotoxin) [147]. Lipid A is the pathogen-associated molecular pattern (PAMP) of LPS that is recognized by TLR₄. Interestingly, immune responses of humans to LPS varies depending on the source of bacterial LPS. Indeed, LPS can be structurally different between gram negative bacteria species and induce distinct immune signaling responses [148].

8.1.2 LPS in humans

The human colon contains approximately 1 kg of bacteria which can produce LPS. As, the outer membrane of bacteria is constantly renewed to maintain integrity, bacteria continuously release LPS in their environment. In the gut of healthy individuals, LPS is safely maintained and lipid A is detoxified by the intestinal phosphatase enzyme. Very low amounts of LPS (up to 5 pg/ml) is normally present in the blood of healthy individuals without inducing any side effects [149]. The human gut is the main reservoir of LPS and an important place for LPS entrance into the blood circulation. During the prandial state, the lipid part of LPS is integrated into chylomicrons and absorbed leading to circulating LPS in the bloodstream [150]. The liver plays a central role in the elimination of LPS through the activation of Kupffer cells [151]. Various liver diseases such as viral hepatitis infections or alcohol-related diseases can lead to increased intestinal permeability (dysbiosis) and thus endotoxaemia. The term endotoxaemia is used when endotox-

ins are present in the bloodstream. Endotoxaemia has also been reported in individuals with obesity, diabetes, chronic fatigue, stress and other conditions [152]. Humans are extremely sensitive to LPS, even at low concentrations. Symptoms of sepsis occur following an exposure to 1-5 ng/kg of LPS and with the lethal dose (septic shock) of LPS being as low as 1 to 2 g [153, 154]. Despite being a strong immunostimulant that can lead to inflammation, fever and septic shock, LPS is also used in the development of vaccines as an adjuvant for its immunostimulatory effect. Low levels of biosynthetically engineered LPS promote TLR activation and improve immunization while limiting its endotoxic activity [155].

8.1.3 Endotoxin tolerance

Prolonged exposure of immune cells to LPS induces LPS tolerance which results in two effects. Repeated administration of LPS leads to immunosuppression that protects the body from cytokine-induced damage. Moreover, LPS-tolerated mice are more protected against a second challenge with invasive pathogens showing that endotoxin tolerance could protect against systemic infections [156].

8.2 TLR₄

TLR₄ was the first TLR to be described. Using genetic evidence, TLR₄ was first proposed as the receptor for LPS in 1998 by Poltorak et al [157]. TLR₄ is composed of an extracellular domain containing a hypervariable domain (HYP) and leucine-rich repeats (LRR), a transmembrane domain (TM) and a cytoplasmic domain named Toll-interleukin-1 receptor (TIR). This domain contains three regions highly conserved across species whereas the extracellular domains exhibit considerable sequence differences. In human blood, TLR₄ is mainly expressed on monocytes and polymorphonuclear cells and its expression significantly increases after LPS stimulation in a dose-dependent manner [158]. In contrast to pDCs which do not express TLR₄, immature monocyte-derived human DCs express low levels of TLR₄. However, its expression is repressed in mature DCs generated with LPS stimulation [159]. B cells also express low levels of TLR₄ [160]. TLR₄ induced signals are mediated through the sequential assembly of the myddosome and the triffosome. However, inflammatory responses to LPS can also occur independently of the TLR₄ pathways through three cellular responses. This includes the LPS response via CD14-dependent endocytosis, the LPS response through caspase 11 and the

LPS detection by the brain-specific angiogenesis inhibitor 1 (BAI1) leading to reactive oxygen species (ROS) production (Figure. 8.1). All these cellular responses induce inflammation to control bacterial spread.

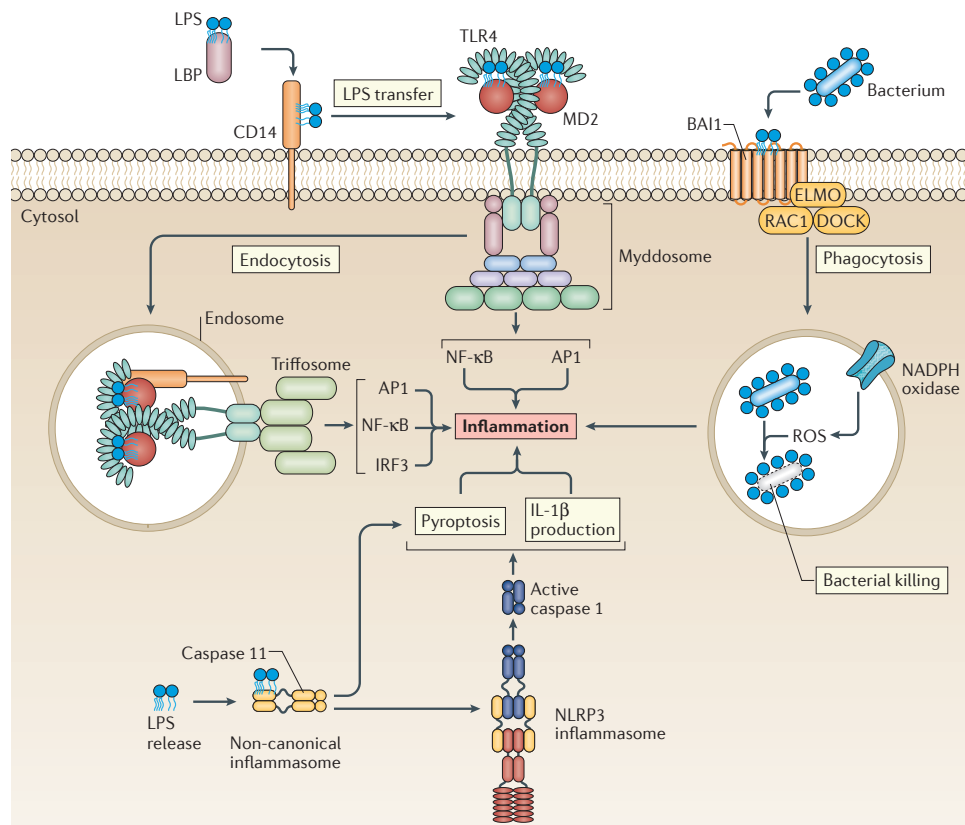


Figure 8.1: **LPS response pathways promoting inflammation [161]**. Schematic representation of the five LPS pathways. LPS is initially sensed by LPS-binding protein (LBP). Next, CD14 delivers LPS to TLR4–MD2 complex leading to the dimerization and activation of TLR4. This process activates the myddosome assembly. Then, CD14 delivers dimerized TLR4 to endosomes which promote signaling through the trifosome. Both pathways result in inflammation. BAI1 protein can also detect LPS at the plasma membrane and promote phagocytosis, inducing the production of reactive oxygen species and inflammation. In the cytosol, LPS is bound by caspase 11 leading to the formation of the non-canonical inflammasome and pyroptosis. In addition, caspase 11 also activates the formation of the canonical NLRP3 inflammasome which induces IL-1 β processing and release.

8.3 LPS responses dependent of TLR4

LPS-binding protein (LBP), CD14 and MD-2 are essential for the interaction of TLR4 with its microbial ligand LPS. LBP is a soluble shuttle protein produced in the liver. It circulates in the bloodstream where it detects lipid A and forms a high-affinity complex

with LPS micelles. LBP alters the membrane to convert them to monomers for delivery to CD14. Then, CD14 concentrates LPS and transfers it to the non-covalently bound TLR₄-MD2 heterodimer complex. This results in crosslinking of two TLR₄ proteins. This homodimerization step sequentially activates two distinct signaling pathways by recruiting adaptors through interactions with the TIR domain. An early MyD88-dependent response controlled by the TIRAP-MyD88 proteins will initiate the inflammatory response followed by a delayed TRIF-dependent response controlled by TRAM-TRIF adaptor proteins [162].

8.3.1 MyD88-dependent pathway (myddosome complex formation)

Dimerization of TLR₄ induces the recruitment of both the sorting adaptor TIRAP (toll/interleukin-1 receptor domain-containing adaptor protein) and the signaling adaptor MyD88 (myeloid differentiation factor 88) to the receptor complex. TIRAP contains TIR domains which allow its interaction with TLRs and thus acts upstream of MyD88. TIRAP induces the assembly of an oligomeric complex called the myddosome which is a supra molecular organizing center (SMOC). Briefly, MyD88 recruits and activates IRAK-4 which in turn recruits and activates IRAK-1. Autophosphorylation of IRAK-1 leads to the activation of TRAF-6 adaptor protein through the formation of a complex with UBC13 and UEV1A proteins. This complex induces the activation of TAK-1 which acts as a common activator of both NF- κ B and MAPK pathways. Activation of these pathways leads to the nuclear translocation of the NF- κ B and AP-1 transcription factors to control the gene expression of pro-inflammatory cytokines (Figure. 8.2) [163, 164].

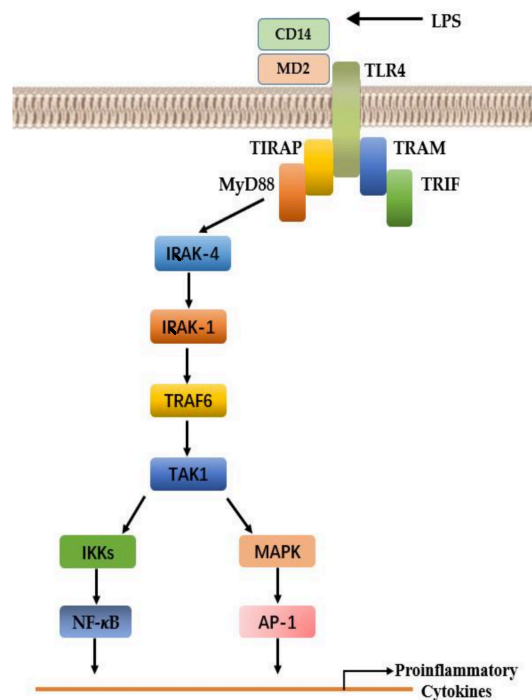


Figure 8.2: **The MyD88-dependent pathway [165]**. Schematic representation of all the molecular actors involved in the MyD88-dependent pathway induced following TLR₄ activation by LPS. This pathway leads to the production of proinflammatory cytokines that contribute to eliminate the infection.

8.3.2 TRIF-dependent pathway (trifosome complex formation)

Once the early response to LPS is triggered, TLR₄ is then internalized into the endosome where a TRIF-dependent pathway is initiated. The sorting adaptor TRAM containing TIR domains (TRIF-related adaptor molecule) is recruited followed by the signaling adaptor TRIF (TIR-containing adapter molecule). TRIF recruits proteins to form a SMOC called the trifosome. TRIF activates both RIP₁ and TRAF₃. RIP₁ mediates downstream nuclear translocation of AP-1 and NF- κ B transcription factors whereas TRAF₃ associates with TANK, TBK₁ and IKK_i to trigger the dimerization and translocation of IRF3 and consequently IFN β gene expression (Figure. 8.3) [162].

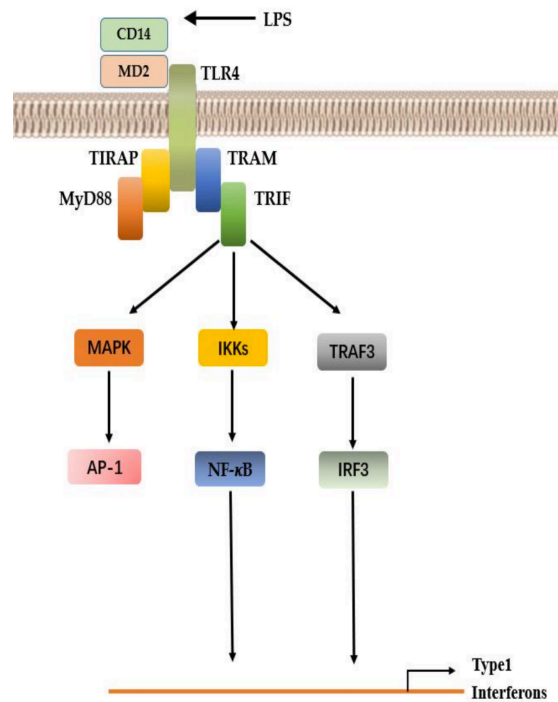


Figure 8.3: **The TRIF-dependant pathway [165]**. Schematic representation of all the molecular actors involved in the MyD88-independent pathway triggered following LPS-induced TLR₄ internalization. This pathway predominantly induces IFN β cytokine production.

8.4 LPS responses independent of TLR₄

8.4.1 LPS induced endocytosis by CD₁₄

CD₁₄ is crucial for TRIF-mediated interferon production [166] as it regulates TLR₄ endocytosis [167]. However, recent studies have highlighted the capacity of CD₁₄ to act as a PRR triggering a TLR₄-independent signaling pathway. Endocytosis of LPS and CD₁₄ proceeds in cells that lack TLR₄. This pathway induces the activation of SFKs (Src-family kinase) and PLC γ 2 (phospholipase γ 2) as well as influx of extracellular calcium leading to NFAT translocation [167, 168, 169]. This pathway is only active in DCs, and not macrophages, and is necessary to induce terminal DC differentiation and apoptotic death which is crucial to maintain self-tolerance and prevent autoimmunity [170].

8.4.2 LPS-induced activation of ROS synthesis and phagocytosis by BAI₁

LPS can also be detected at the plasma membrane by BAI₁. In contrast to other LPS receptors, BAI₁ detects the core part of LPS which induces the recruitment of the ELMO and DOCK proteins. This complex activates the RAC₁ enzyme which promotes phagocytosis and the assembly and activation of NADPH oxidase leading to ROS production and inflammation [171, 172].

8.4.3 LPS-induced assembly of inflammasomes by caspase 11

Detection of LPS can also occur in the cytosol especially when pathogens disrupt phagosomal membranes or with the action of GBP (guanylate binding proteins) induced by interferons which disrupt the vacuole membrane integrity [173]. Caspase 11 detects intracellular LPS and cleaves GSDMD (gasdermin D) which leads to the formation of the GSDMD pore. This pore promotes pyroptosis and the release of IL-1 β . In parallel, caspase 11 also activates the assembly of the NLRP₃ inflammasome. The active caspase 1 cleaves pro-IL-1 β which induces inflammation [174, 175, 176, ?].

8.5 Regulation of TLR₄ signaling pathway

Improper regulation of the LPS-TLR₄ pathway can induce massive inflammation and cause chronic inflammatory disorders or even sepsis. For instance, septic shock can be the result of altered negative regulation of the pathway (leading to excessive inflammation) as much as activating signals [177, 178].

8.5.1 Positive regulation of TLR₄ signaling pathway

The integrin CD11b has been shown to be a positive regulator of both TLR₄ signaling pathways on DCs, but not on differentiated macrophages. DCs express lower levels of TLR₄ receptor and CD14 co-receptor than macrophages, thus CD11b promotes the formation of TLR₄ signaling pathways in DCs but not in macrophages. In the absence of CD11b, LPS binding to TLR₄ is impaired leading to less induction of T cell responses. CD11b promotes TIRAP enrichment of the MyD88-dependent pathway and facilitates TLR₄ endocytosis of the TRIF-dependent response [179].

8.5.2 Negative regulation of TLR₄ signaling

Inhibition of the TLR₄ pathway is essential to protect the host from damage caused by persistent inflammation. Many proteins which inactivate multiple levels of the TLR₄ pathway have been described and mechanisms of inhibitory action are diverse (Figure. 8.4). These regulators can inhibit the association between molecules by interacting with one of the signaling proteins, they can also promote their degradation or downregulate their activation. Some proteins can be produced after LPS stimulation providing a negative feedback loop to stop TLR₄ signaling pathways. Several of these negative regulators are implicated in the process of endotoxin tolerance defined as a reduced responsiveness to a second LPS challenge after a first exposition to endotoxin. LPS desensitization can alter resistance to viral infection (by reducing the level of interferon production after challenge) or increase resistance to bacterial and fungal infections (through the boost of TNF production during LPS pre-treatment) [180].

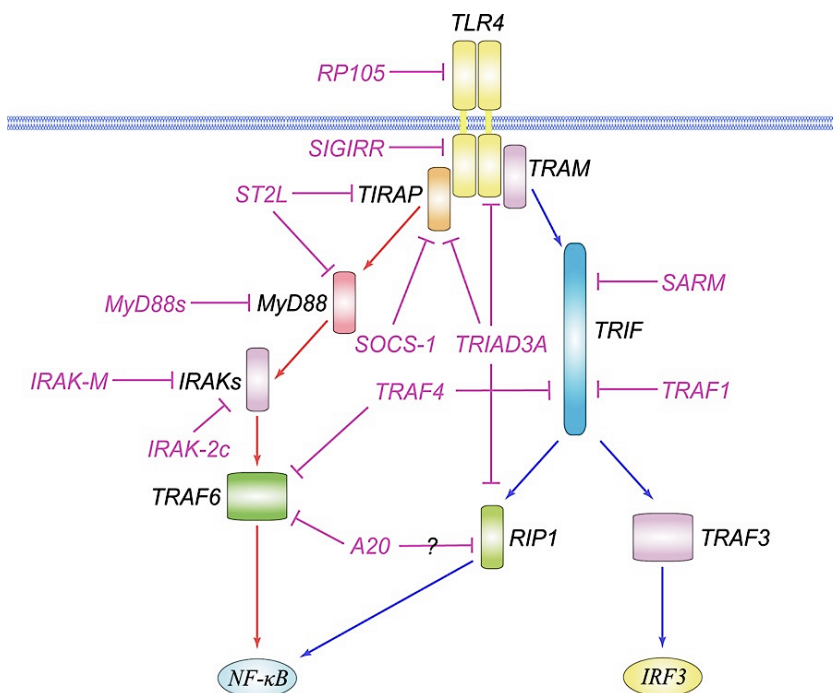


Figure 8.4: **Negative regulation of TLR₄ signaling pathways [163].** Negative regulators (in pink) targets multiple levels of the TLR₄ signaling pathway through different mechanisms.

9. IL-12p70 and type I IFN cytokine secretion in bacterial and viral infection

9.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (*M. tb*) is an intracellular pathogen responsible for tuberculosis (TB) that mainly infects macrophages and affects the lung. TB germs are spread between individuals through the air and the infection results in two different clinical outcomes which include asymptomatic latent infection (LTBI) or active TB disease. LTBI individuals cannot transmit the disease but 5 to 10% of them will develop active disease [181]. Tuberculosis is a major global health threat as it is estimated that a quarter of the world population is infected with *M. tb* and 10 million people progress into active TB disease each year [182]. Tuberculosis is preventable in children with BCG vaccination and curable with a 6-month treatment comprised of the combination of 4 antibiotics. However, a significant number of drug-resistant cases arise due to inappropriate use of drugs or premature treatment interruption [183, 184]. Two tests have been developed to detect tuberculosis infection. The first one is the tuberculin skin test which require two visits, intradermal injection and can lead to false positive results in people that are vaccinated with BCG. The other test is a blood-based test in which immune cells are stimulated with *M. tb* antigens and IFN γ production is measured by ELISA or ELISPOT (QuantiFERON-TB Gold assay and T-SPOT TB test respectively) [185]. Both tests do not allow to distinguish between asymptomatic latent infection and active TB disease and additional tests are required such as microscopy or PCR to diagnose active TB disease. Thus, there is an urgent need for innovative or improved diagnostics to (i) identify new biological markers that allow to discriminate LTBI from active TB, (ii) predict progression from latent to active TB disease and (iii) anticipate treatment success or failure. In addition, new therapies are critically needed for drug-sensitive and multidrug-resistant tuberculosis [186].

9.1.1 Type I IFNs and IL-12p70 in *M. tb* disease

Several cytokines such as TNF α , IL-1, IL-12p70 and IFN γ contribute to host protection against *M. tb* [187, 188, 189]. In the host, *M. tb* triggers multiple cytosolic and cell surface PRRs inducing type I IFNs including TLR4 and its downstream triffosome complex. However, the role of type I IFNs in the immune response to *M. tb* has been very controversial in the field [190]. Type I IFNs can display both protective or detrimental functions depending on contextual factors and settings of the host-pathogen encounter. A type I IFN gene signature was defined in the blood of patients with active TB disease and in 10 to 25% of latently infected individuals [191, 192]. Overexpression of ISGs occurs specifically in neutrophils, to a lesser extent in monocytes and is associated with disease severity [191]. This suggests that high and sustained levels of type I IFNs has a detrimental role in TB. This is supported by additional genetic studies. As an example, a genetic variation in the human IFNAR1 gene leading to impaired type I IFN signaling was associated with increased resistance to TB [193]. Thus, these results suggest a harmful role of high and sustained production of type I IFNs and suggest that type I IFNs promotes both bacterial proliferation and disease pathogenesis. The mechanisms of exacerbation of *M. tb* by type I IFNs are not completely understood yet but studies have shown that high levels of type I IFNs are associated with a decreased production of proinflammatory cytokines such as IL-12p70 which impairs the Th1 response [194, 195]. Although the detrimental functions of type I IFNs in TB has been widely studied, type I IFNs can also display a protective role under specific conditions. Administration of IFN α with antimycobacterial treatment to recurrent active TB patients or patients who were not responsive to standard therapy reduced bacterial burden and improved clinical symptoms [196, 197]. In addition, type I IFNs have been reported to increase host resistance against some avirulent mycobacterial strains [198, 199]. Benefits of IFN α have also been shown in infected patients and mouse models with IFNGR signaling deficiencies [200, 201]. The dominant inhibitory effect of type I IFN on IFN γ may hide some protective effects of type I IFNs. Finally, early type I IFN production is important to control the infection as they prime innate immune cells which enhance the secretion of proinflammatory protective cytokines. Indeed, pretreatment of macrophages with type I IFNs before *M. tb* infection induces the production of IL-12p70 and TNF α [202]. Thus, the timing, the *M. tb* strain and the activity of the IFN γ pathway may be key for protective effects and effective immune responses of type I IFNs in tuberculosis [203]. IL-12p70 and IFN γ -mediated T cell functions have an essential role in controlling *M. tb* infection. Patients who suffer

from MSMD which result in a defect in IL-12p70 or IFN γ signaling pathways, are highly susceptible to mycobacterial infection including *M. tb*. IFN γ production mediated by IL-12p70 activates macrophages to produce protective cytokines inducing intracellular killing of *M. tb* via the production of nitrogen species and reactive oxygen [190].

9.2 Hepatitis C viral infection

HCV is a positive single-stranded RNA virus that causes liver disease. HCV transmission typically occurs through direct exposure to infected blood and, the virus can induce both acute and chronic infections. 15 to 45% of infected people spontaneously clear the HCV infection without treatment within 6 months post infection. However, the majority of people infected with HCV (70% to 80% of individuals) develop chronic infection [204]. Hepatitis C is a major public health problem as it is estimated that more than 71 million individuals are chronically infected with HCV. 10 to 20% of chronically infected individuals develop complications leading to progressive liver damage which may induce cirrhosis and hepatocellular carcinoma within 20-30 years resulting in more than 450,000 annual deaths. So far, 8 genotypes have been identified with genotypes 1, 3 and 4 being the most common ones [205]. Effective treatments exist to cure HCV infection. For more than 15 years, IFN α associated with ribavirin were used to treat hepatitis C resulting in 75 to 90% of cured patients [206]. Since 2014, highly effective direct acting antiviral agents (DAAs) have been approved that can cure HCV in more than 95% of the patients. However, it is estimated that only 20% of the persons are aware of their infection and 15% of them are treated [205]. Universal access to both DAAs therapy and affordable diagnostics are key to achieve the elimination of the virus [207]. The infection can be diagnosed through the detection of hepatitis C antibodies which arise within 12 weeks of infection or with the detection of viral RNA. The detection of RNA at 12 weeks post exposure predicts the chronicity of the disease and indicates the necessity for treatment [208]. The use of DAAs is unlikely to achieve complete HCV elimination without an effective vaccine that can limit viral transmission and HCV reinfection. So far, no safe and protective vaccine against HCV is available for clinical use. Only two vaccine candidates have reached the phase I testing with one reaching phase II clinical trials [209]. A better understanding of protective immune responses and is a crucial for the development of a highly effective vaccine.

9.2.1 Immune responses to HCV: role of type I IFNs and IL-12p70 cytokines

During acute infection, the elimination of the virus is associated with a rapid induction of type I and III IFNs that are produced by infected cells, macrophages and DCs [210, 211]. HCV induces TLR pathways (TLR3, TLR4, TLR7 and TLR8) leading to the activation of NF- κ B, IRF3 and IRF7 transcription factors [212, 213]. TLR recognition induces the production of type I IFNs in the early phase of acute infection and then, NK cells, Th1 and cytotoxic T cells produce type II IFN during the late phase of acute infection [214, 215]. A broad CD4⁺ T cell response and proliferation associated with IFN γ , IL-2 and TNF α production are observed in the acute phase of HCV infection of the patient who spontaneously clear the virus [216, 217, 218]. Thus, IFNs are the essential cytokines for the innate immune response to HCV as they are responsible for the activation of an antiviral state through the transcriptional activation of genes that are predominantly ISGs [219]. However, HCV can escape IFN-mediated innate immunity via an elaborate set of mechanisms that are not entirely fully understood but might include blockade of ISG protein translation, viral replication in cell compartments that are not accessible by IFN and viral protein antagonist mechanisms of action [220]. For example, HCV produces a protease that cleaves and inactivates both mitochondrial antiviral signaling (MAVS) molecule and TRIF protein which induces IFN β [221, 222]. In the majority of infected individuals, HCV persists and the infection becomes chronic. A delayed induction of adaptive immune responses is observed in chronically infected patients. It is proposed that a prolonged innate immune activation by HCV results in an impaired development of an appropriate adaptive immune response [223]. In addition, a chronic infection is characterized by altered NK and T cell responses which are unable to clear the virus and a type I IFN signature that correlates with the severity of HCV [224]. Of note, persistence of high ISG expression is the strongest negative predictor of the response to the IFN α -based treatment suggesting that the constant activation of the IFN response makes it refractory to additional stimulation [225, 226]. Levels of IL-12p70 in the context of HCV infection with and without treatment, have been investigated in several studies. All studies highlight a significant elevation of IL-12p70 concentration in the serum of HCV infected individuals compared to healthy subjects and IL-12p70 levels were significantly reduced following the co-administration of IFN α and antiviral therapy [227, 228, 229]. Moreover, high levels of IL-12p70 in the serum of chronic HCV infected individuals have been linked with sustained virological response (SVR)

after treatment with IFN α showing the beneficial effect of IL-12p70 to cure HCV using the interferon-based therapy [229, 228, 230].

Part II

Objectives

10. Hypothesis and aims of the project

Despite its essential role in combating both bacterial and viral infection, we have observed highly variable IL-12p70 responses in the 1,000 healthy individuals of the MI cohort, as well as in patients infected with *Mycobacterium tuberculosis* and hepatitis C virus. Following whole blood LPS stimulation of 25 donors of the MI cohort, 24% of these healthy donors failed to secrete IL-12p70 [23], but its production was restored with interferon alpha (IFN α) co-stimulation (Figure. 10.1).

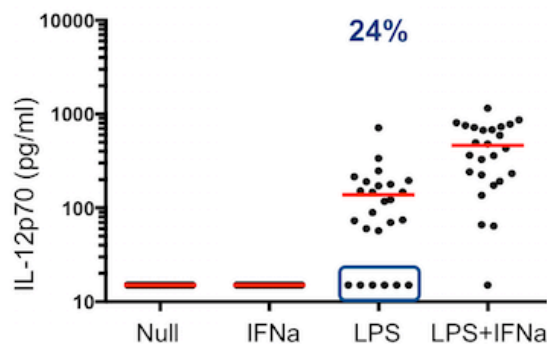


Figure 10.1: IL-12p70 cytokine secretion in healthy individuals. Whole blood from healthy individuals was stimulated for 22 hours in TruCulture tubes with no stimulus (null), LPS, IFN α or LPS+IFN α stimuli. IL-12p70 cytokine levels from the plasma were measured using Luminex technology.

We hypothesize that dissecting this variance in the healthy MI reference population will provide new insights into disease pathogenesis. Testing this hypothesis requires first to characterise the IL-12p70 phenotype (**Aim 1**) and define (i) whether the healthy donors for whom IL-12p70 was not detected by Luminex under LPS stimulation are low or non-responders, (ii) if the phenotype is stable over time, (ii) which immune cell types produce IL-12p70 and IL-23 cytokines in whole blood of healthy donors after LPS stimulation. For the second aim (**Aim 2**), we took advantage of data available in the MI LabKey database for the 1,000 healthy donors that includes flow cytometry (including immune cell counts and mean fluorescence intensity (MFI)), CpG methylation sites, genotyping of SNPs, and transcriptomic data. We analyzed these data sets to better understand IL-12p70 inter-individual variability within healthy donors upon LPS stimulation. Fur-

thermore, we investigated if this phenotype shows any environmental associations as captured by the electronic case report form (eCRF). IL-12p70 production in response to TLR activation is dependent on production of endogenous type I IFNs which regulate IL-12p35 mRNA accumulation [53]. This finding explains why the co-stimulation with LPS and IFN α induces secretion of IL-12p70 in healthy donors who do not respond to LPS stimulation alone. Specifically, type I IFNs induce *IL12A* gene expression (which encodes for the p35 subunit) allowing IL-12p70 secretion. Moreover, some studies have shown that a first wave of IFN γ is necessary to induce IL-12p70 [231, 232, 233]. Also, IFN γ induced by IL-12p70 further enhances IL-12p70 secretion creating a positive feedback loop [50]. Thus, based on this literature, we aim (**Aim 3**) to better dissect the relationship between type I and II interferons and IL-12p70 secretion in human whole blood, and so better understand potential upstream regulators of cytokine variability. Finally, we validated our findings in both HCV and TB cohorts.

Part III

Results

II. Results

II.I Variation in the IL-12p70 response upon TLR4 activation in healthy population is altered in viral and bacterial infection

We initially observed high levels of inter-individual variability in IL-12p70 cytokine responses after whole blood LPS stimulation in 25 healthy donors of the *Milieu Interieur* (MI) cohort [23]. To assess IL-12p70 cytokine secretion in viral and bacterial infections, ultrapure lipopolysaccharide (LPS) was used to stimulate whole blood from patients chronically infected with *Mycobacterium tuberculosis* (*M. tb*) and hepatitis C virus (HCV) using TruCulture tubes. IL-12p70 cytokine secretion was measured using a Luminex assay. IL-12p70 secretion from patients infected with *M. tb* or HCV was significantly lower than in healthy donors, suggesting that the Th1 response is altered in disease (Figure. II.IA). To further characterize potential reasons behind this cytokine variability, we examined the 1,000 healthy donors of the MI cohort, for whom whole blood was stimulated for 22 hours with LPS (TLR4 agonist), Poly(I:C) (TLR3 agonist), and an unstimulated control (null). Although an IL-12p70 response was detected for all donors upon TLR3 activation, 28% of healthy donors did not exhibit detectable levels of IL-12p70 upon TLR4 stimulation. This demonstrates that the variation observed for IL-12p70 is specific to whole blood TLR4 induced responses (Figure. II.IB). As IL-12p70 is a heterodimer composed of IL-12p35 and IL-12p40, which also forms IL-23 (with IL-12p19), we examined whether these responses were also impacted after LPS stimulation. Measurement of IL-12p40 (Figure. II.IC) and IL-23 (Figure. II.ID) in LPS stimulated supernatants revealed a normal distribution of responses suggesting that this observed variability was specific for the IL-12p70 heterodimer cytokine. To test whether the 28% of MI donors secreted either low or no amounts of IL-12p70 upon LPS stimulation, we developed an ultrasensitive Simoa digital ELISA. This assay has a limit of detection (LOD of 0.01 pg/ml), which is 1000 times more sensitive than Luminex (LoD: 18.5 pg/ml). Using this novel assay, we re-defined the donors as low responders as IL-12p70 was detectable for the 28% of healthy donors after LPS stimulation compared with the null condition

($P < 0.0001$) (Figure. II.IE). To test if this phenotype was stable over time, we measured IL-12p70 in 500 MI donors at a second time point, 2 to 6 weeks later. The IL-12p70 responses over the 2 timepoints showed a strong and significant correlation ($R = 0.71$, $P < 0.0001$) (Figure. II.SIA), showing that our approach allows the study of stable and robust phenotypes. Moreover, the IL-12p70 response was also significantly different ($P < 0.0001$) between the two groups as initially observed (Figure. II.IF). We also confirmed that this difference was evident after *E. coli* stimulation (Figure. II.SIB), and that results for LPS and *E. coli* with Luminex (Figure. II.SIC) and Simoa (Figure. II.SID), were consistent and reproducible. Overall, these results indicate that inherent variability in the IL-12p70 response after TLR₄ activation is consistent and prevalent.

II.I. Variation in the IL-12p70 response upon TLR4 activation in healthy population is altered in viral and bacterial infection

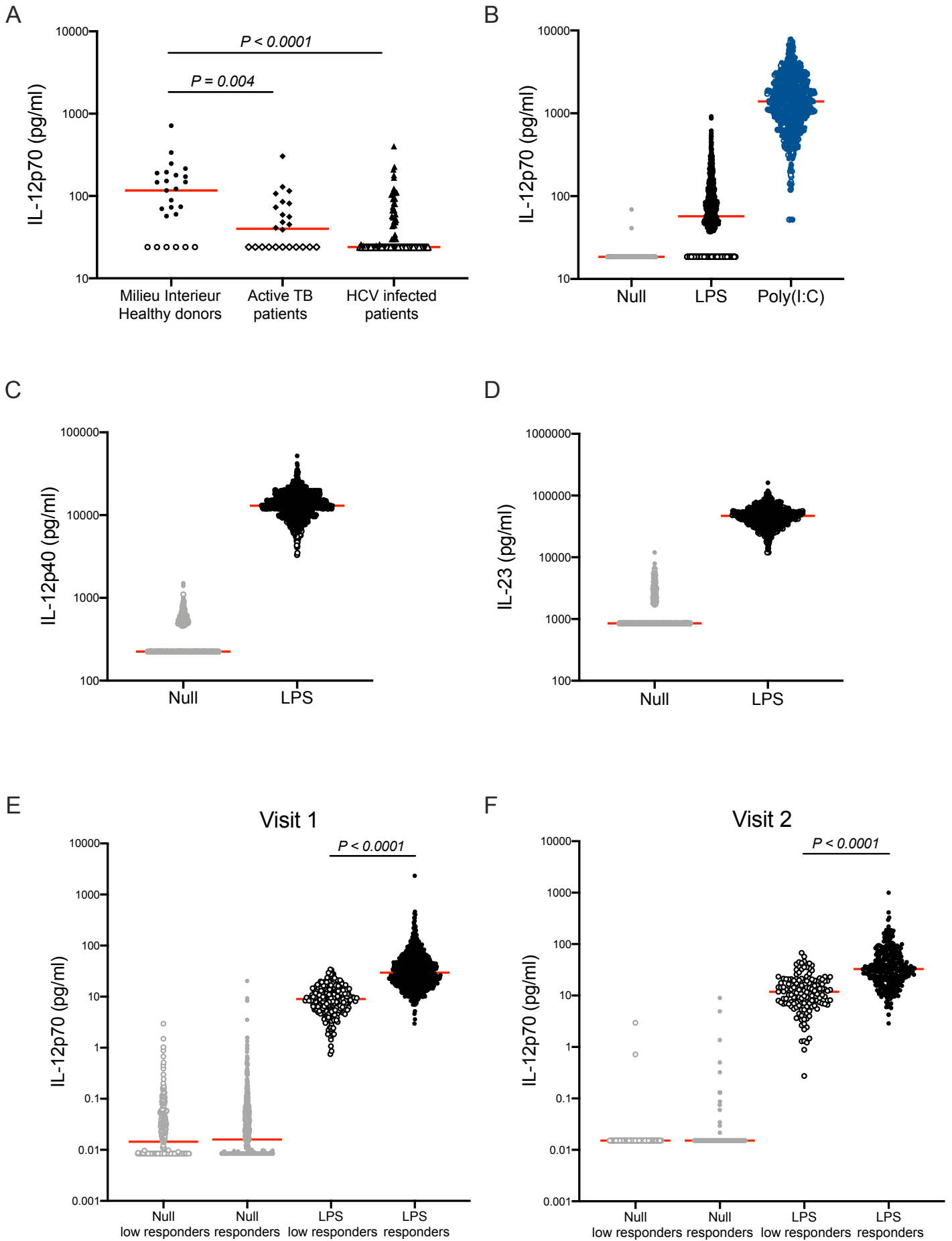


Figure 11.1 (previous page): **IL-12p70 variability in healthy donors and in patients infected with *M. tb* or HCV.** (A) IL-12p70 measured using Luminex after stimulation of whole blood with LPS for 22h from healthy donors (n=25), patients infected with active TB (n=24) or chronic HCV (n=100). P values were determined by Mann-Whitney tests. (B) IL-12p70 measured by Luminex in healthy donors (n=1,000) after null, LPS and Poly(I:C) stimulation. (C) (D) IL-12p40 and IL-23 measured by Luminex in healthy donors (n=1,000) after null and LPS stimulation. (E) (F) IL-12p70 secretion measured by Simoa in the two groups of *Milieu Interieur* IL-12p70 responders after null and LPS stimulation at two separate time points (V1 n=1,000; V2 n=500). Red lines indicate the median value for each group. P values were determined by an unpaired Student's t test on log10 values. Empty symbols represent IL-12p70 low responders as defined upon LPS 22 hours whole blood stimulation, full symbols represent IL-12p70 responders.

11.2 Low IL-12p70 responders have less activated monocytes and DCs

To take advantage of existing cellular data sets [20], we first defined the immune cells that produce IL-12p70 in whole blood after LPS stimulation. Whole blood of 10 healthy donors was stimulated with LPS for 22 hours in the presence of a protein transport inhibitor 8 hours after the start of the stimulation. A combination of 10 surface antibodies was used to characterize the major circulating immune cell populations. As the IL-23 cytokine shares the p40 subunit with IL-12p70, both IL-12p70 and IL-23 were assessed intracellularly in the 8 studied cell types. The null condition was used to determine the positivity threshold for IL-12p70 and IL-23 secretion (Figure. 11.2A). We observed that CD14⁺ monocytes were the main producers of IL-12p70 cytokine upon LPS stimulation, followed by DCs (Wilcoxon test, FDR < 0.05). Interestingly, more than 30% of CD14⁺ monocytes secreted both IL-12p70 and IL-23 together, whereas around 7% of CD14⁺ monocytes secreted IL-12p70 only at this time point. A similar observation was made with conventional dendritic cells 2 (cDC2) (Figure. 11.2B). Thus, the majority of cells producing IL-12p70 also secrete IL-23. We then explored whether the low responders of the MI cohort had less circulating CD14⁺ monocytes or DCs than the responders at steady state. Immunophenotyping of the 1,000 subjects was previously performed using ten flow cytometry panels and 166 distinct immunophenotypes were reported [20]. This includes 76 absolute counts of circulating cells, 87 expression levels of cell surface markers and 3 cell ratios. No significant differences between the two groups regarding the CD14⁺ monocytes, cDC2 and plasmacytoid dendritic cells (pDCs) absolute cell counts were observed (Figure. 11.2C). However, applying an unbiased analysis to the entire immunophenotyping dataset, revealed significantly (FDR < 0.05) lower cell surface expression of HLA-DR and CD86 on CD14⁺ monocytes and HLA-DR on cDC2 in the low re-

sponders as compared to the responders (Figure. 11.2D). Collectively, our analysis shows that CD14⁺ monocytes and DCs produce both IL-12p70 and IL-23 upon LPS stimulation, and these cells expressed less HLA-DR and CD86 activation markers in the low responder group.

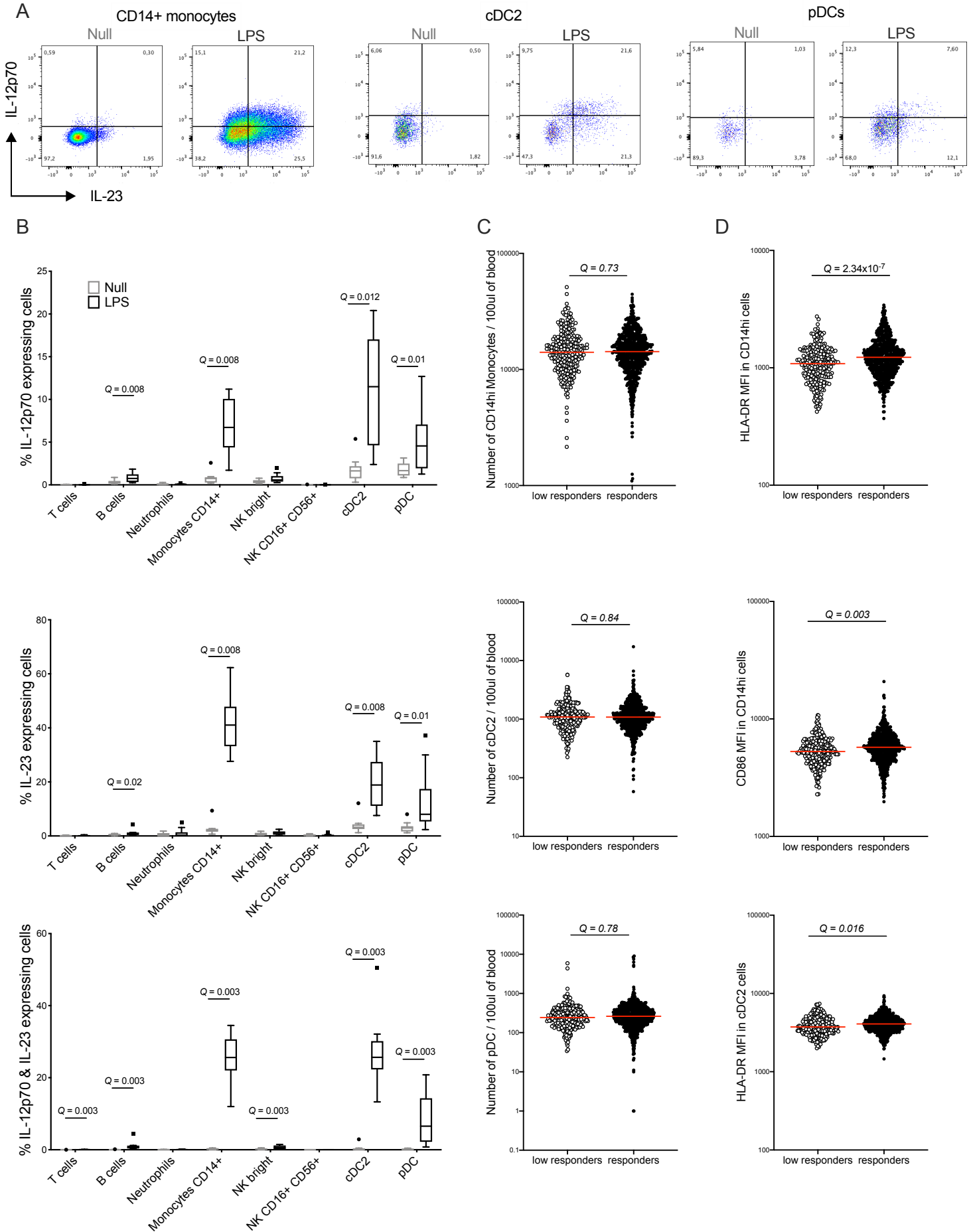


Figure 11.2 (previous page): **CD14⁺ monocytes and dendritic cells secrete IL-12p70 after LPS stimulation.** (A) Representative dot plots of IL-12p70 and IL-23 secretion upon null and LPS 22h stimulation measured by flow cytometry in CD14⁺ monocytes, cDC2 and pDCs. (B) Tukey box-whisker plots of the percentage of IL-12p70 and/or IL-23 cytokine positive cells 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. (C) Numbers of CD14^{hi} monocytes, cDC2, and pDCs for the responder and low responder groups (n=1,000). (D) 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 (C) and 88 (D) multiple comparison testing (n=1,000).

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

Next, we investigated whether genetic differences could explain this phenotype utilizing existing genome-wide DNA data sets [20]. Protein quantitative trait loci analysis was performed to find associations between IL-12p70 secretion measured by Simoa after 22 hours LPS stimulation and 5,265,361 genotyped single nucleotide polymorphisms (SNP). By mapping local cis-acting pQTLs (within 1 Mb of each gene coding for the *IL12A* and *IL12B* genes), we found significant associations between five variable genetic regions, SNPs at rs17753641, rs17809756, rs17810546 and rs76830965 and indel at rs143060887 and IL-12p70 levels ($P < 4.32e-6$) (Figure. 11.3A). The group of donors having homozygous major allele for these 5 genetic regions had a significantly higher proportion of IL-12p70 low responders compared to the heterozygous genotype group ($P < 0.0004$). These five SNPs are all located near the *IL12A* gene and found to be in 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 we found that the variants rs17753641 and rs76830965 remained significantly associated with IL-12p70 secretion following LPS stimulation (data not shown). Importantly, none of these SNPs impacted IL-12p40 or IL-23 secretion (representative example of rs143060887 shown in (Figure. 11.3B) and (Figure. 11.S3A)). We also explored whether these 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 LPS 22 hours stimulation. We found that the allelic dosage of the five SNPs did not impact the expression of *IL12A*, *IL12B* and *IL23A* genes coding for p35, p40 and

p19 subunits respectively ((Figure. II.3C) and (Figure. II.S3B)). Together, our analysis indicated that five cis-acting variants are associated with less IL-12p70 secretion but not gene expression after 22 hours LPS stimulation in healthy donors.

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

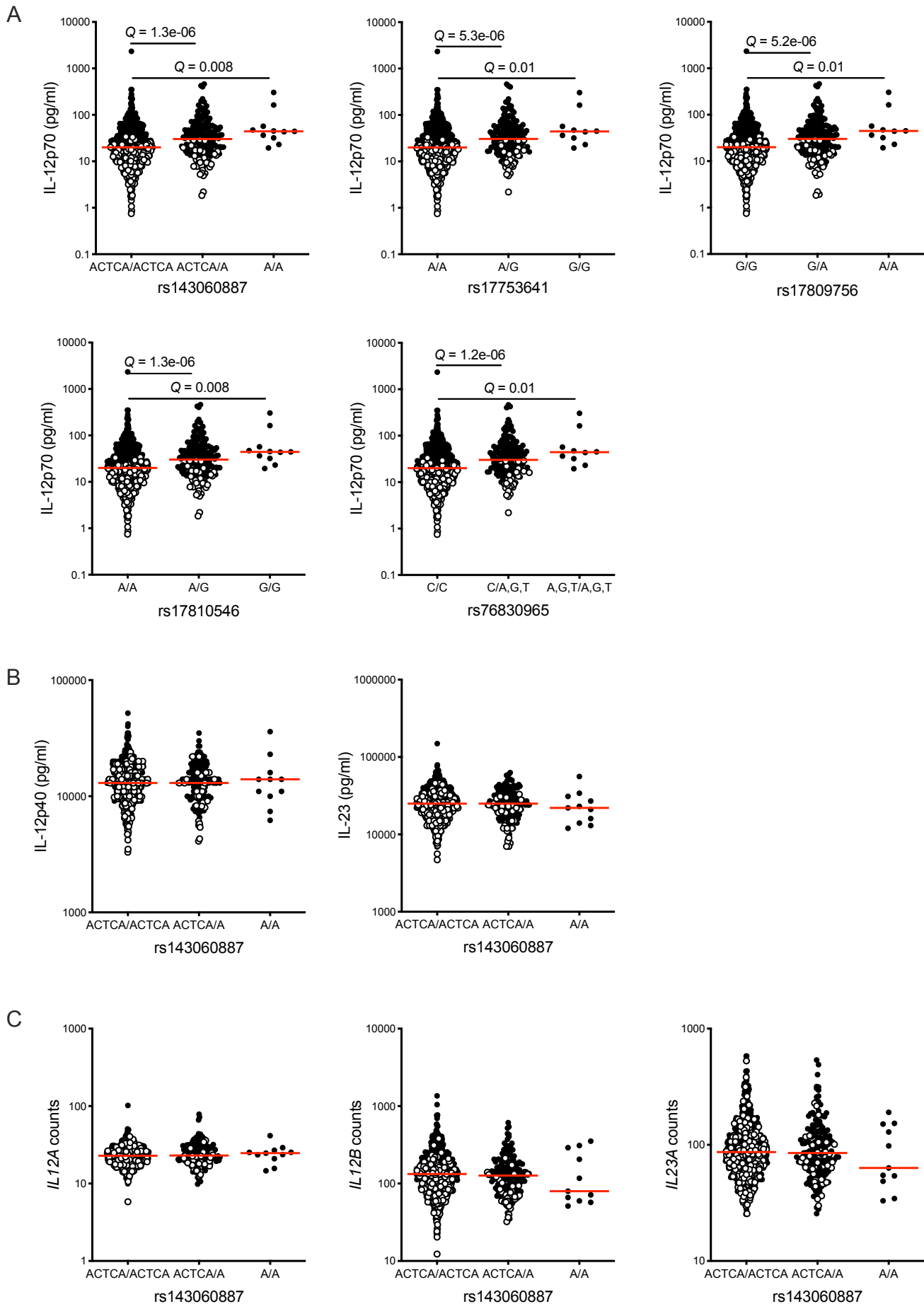


Figure II.3 (previous page): **LPS-specific cis-acting pQTLs on IL-12p70 production.** (A) Local pQTLs located near the *IL12A* gene acting specifically on IL-12p70 protein secretion measured in the 1,000 *Milieu Interieur* donors in response to 22h LPS stimulation. (B) IL-12p40 and IL-23 protein secretion after 22h 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 22h 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.

II.4 Variability in IL-12p70 is determined by upstream variability in IFN β

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 51 differentially expressed genes (FDR < 0.01 with a maximum fold change value of 1). However, following LPS stimulation 332 genes were significantly differentially expressed (Figure. II.4A). We found that gene expression of the three subunits was significantly lower ($P < 0.0001$) in the low responder group compared with the IL-12p70 responders after LPS 22 hours stimulation (Figure. II.S4A) showing that the levels of subunit transcripts is responsible for the concentrations of cytokine observed. Interestingly, IFN γ was the most differentially expressed gene with higher expression in the responders (FDR = $7.34e-70$ and fold change = 1.51). In addition, gene set enrichment analysis showed that 74 of the 332 differentially expressed genes were involved in the IFN γ response pathway (FDR = 0.02) (Figure. II.S4B). Next, we performed proteomic analysis between the responder and low responder groups to determine whether other proteins were differentially secreted between the two groups following LPS stimulation. We found that the secretion levels of 16 proteins (out of 25 measured), other than IL-12p70, were significantly different between the low responders and the responders with CXCL5 (ENA78) being the only protein significantly more secreted in the low responder group (Q value < 0.01) (Figure. II.4B). Previous studies have reported that a first wave of IFN γ is necessary to induce IL-12p70, which triggers IFN γ secretion furthering enhancing IL-12p70 through a positive feedback loop [231, 232, 233]. Moreover, IFN α induces *IL12A* gene expression, that codes for the p35 subunit, in response to TLR activation [53]. To assess the role of type I and II interferons in IL-12p70 secretion upon LPS stimulation we performed a detailed kinetic

11.4. Variability in IL-12p70 is determined by upstream variability in IFN β 61

analysis. Whole blood of 5 healthy donors was stimulated with LPS and IL-12p70 and type I and II interferons were measured at 14 different timepoints (0 to 30 hours) using Simoa. In 4 out of 5 donors, both IFN γ and IFN α were produced either simultaneously or after IL-12p70, suggesting that these two cytokines are not responsible for IL-12p70 secretion. However, for all 5 donors, IFN β secretion began 30 minutes to 1 hour before IL-12p70 induction (Figure. 11.4C). These combined observations suggest a link between IFN β and IL-12p70 secretion after LPS stimulation. To further test this, we performed a time series analysis using a linear mixed-model approach which showed a significant association between IFN β and IL-12p70 (FDR = $6.52e-9$) whereas no significant associations were found with either IFN γ or IFN α (FDR = 0.66) (Figure. 11.4D). In parallel, gene expression was assessed for these 5 donors and the linear mixed-model approach was also applied to explain *IL12A* and *IL12B* gene expression (Figure. 11.4C). While *IL12B* gene expression was correlated with all type I and II interferon genes *IFNA2*, *IFNG* and *IFNB1* genes (FDR = 0.03), *IL12A* expression gene was significantly correlated with *IFNG* and *IFNB1* (FDR = 0.02 and FDR = 0.04 respectively) (Figure. 11.4D). To determine if the low responders of the MI cohort secreted less type I IFNs, we measured IFN α and IFN β proteins in null and LPS TruCulture supernatants after 4 hours of stimulation in a subset of 100 donors. Both IFN α and IFN β proteins were significantly lower in the low responder group, although IFN β was secreted to a much higher degree than IFN α (about 2 million times more) (Figure. 11.4E). Furthermore, early IFN β secretion was positively and significantly correlated with later induction of IL-12p70 (Pearson's R value 0.63, $P < 0.0001$) (Figure. 11.4F). Together, our analysis indicates that low secretion of IFN β is responsible for the IL-12p70 low responder phenotype in whole blood of the healthy donors after LPS stimulation.

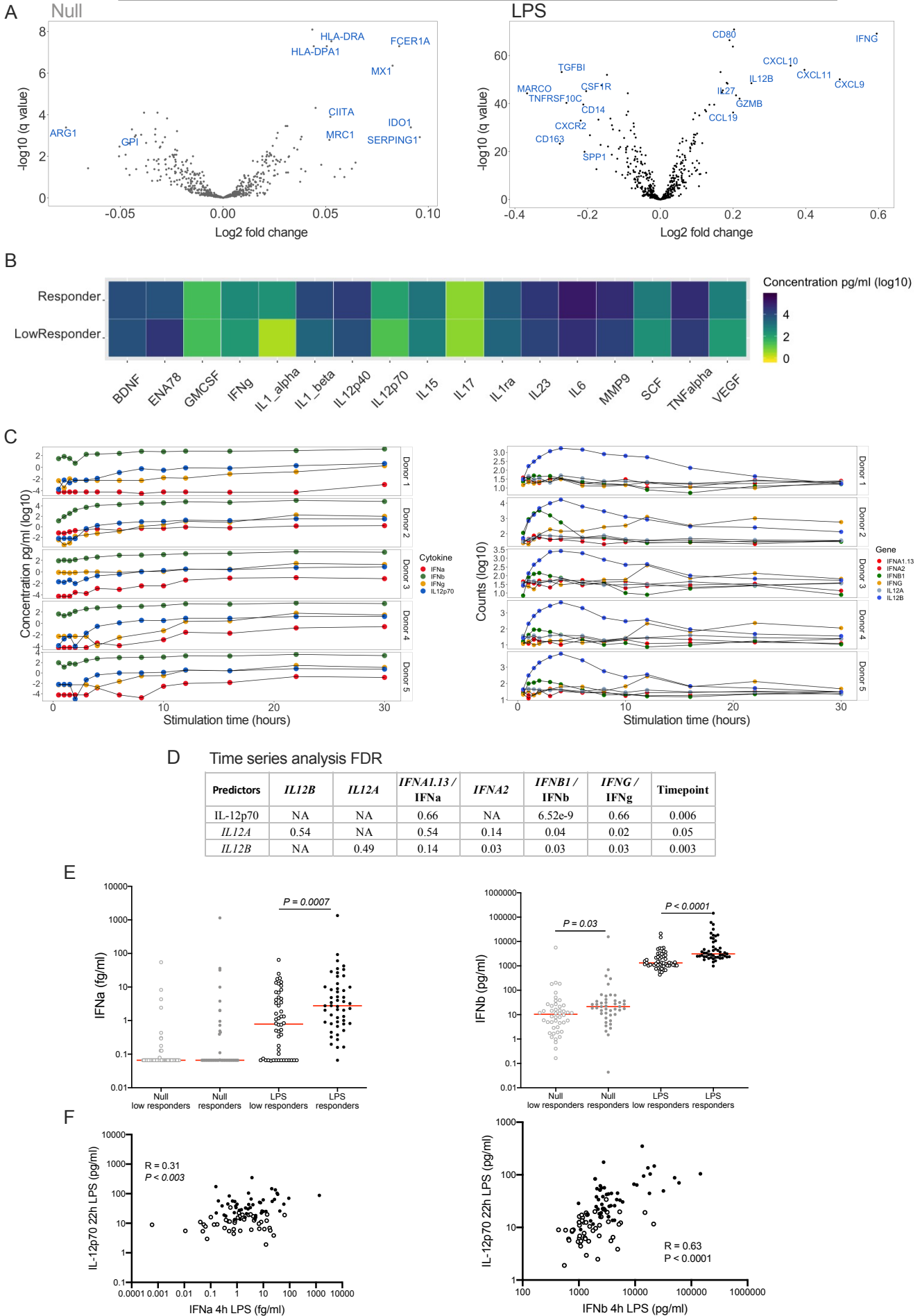


Figure 11.4 (previous page): **Kinetic responses and interactions of type I and II interferons and IL-12p70.** (A) Volcano plots displaying differentially expressed genes between *Milieu Interieur* IL-12p70 low responders and responders upon 22h null and LPS stimulation. Negative log₂ fold change represents genes that are more expressed in low responders whereas positive log₂ fold change represents genes that are more expressed in responders. The cut offs 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) Heatmap representing the proteins for which the production was significantly different between the low responders and the responders in the 1,000 *Milieu Interieur* donors following LPS stimulation (Q values < 0.01). Q values were determined by an unpaired Student's t test using log₁₀ values and false discovery rate correction was performed for multiple comparison testing. (C) Type I (IFN α and IFN β) and II (IFN γ) interferons and IL-12p70 protein secretion and gene expression over time after LPS stimulation for 5 healthy donors. (D) Table reporting FDR Q values of time series analysis on protein secretion and gene expression. (E) Type I IFNs measured by Simoa after 4h 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 MI donors. (F) Correlation between IFN α or IFN β secretion after 4 hours of stimulation and IL-12p70 secretion upon 22 hours LPS stimulation. Pearson correlation tests were performed on log₁₀ values; n=100 MI donors. Empty circles represent IL-12p70 low responders of the *Milieu Interieur* cohort.

11.5 The secretion of IFN β by monocytes is essential for IL-12p70 secretion/Th1 response upon LPS stimulation

To further confirm which type I interferon is crucial for LPS induced IL-12p70 secretion we performed additional whole blood stimulations. Addition of both IFN α and IFN β to LPS stimulation resulted in a significant increase in IL-12p70, however the fold increase was a log (x9.8) greater with IFN β (Figure. 11.5A). Either cytokine given alone did not induce IL-12p70, suggesting that type I IFNs can induce the production of only one of the IL-12p70 subunit. In contrast, the addition of IFN β and IFN α to LPS stimulation did not induce IL-12p40 nor IL-23 secretion compared to the LPS stimulation suggesting that type I IFNs regulate production of the p35 subunit (Figure. 11.5A). We further investigated the regulation of the p35, p40 and p19 subunits by measuring their gene expression following 4 hours stimulation with IFN α , LPS, LPS+IFN α and an unstimulated control (null) in 25 donors of the MI cohort. We observed that IFN α specifically induced *IL12A* but not *IL12B* nor *IL23A* gene expression confirming that type I IFNs up-regulate the production of the p35 subunit (Figure. 11.5B). Interestingly, we found that the addition of IFN α to the LPS stimulation decreased both *IL12B* and *IL23A* gene expression suggesting an inhibitory role of type I IFNs in the production of IL-12p40 and IL-23 cytokines. To determine whether type I IFNs were essential for IL-12p70 produc-

tion, we blocked the type I IFN pathway. Neutralization of the IFNAR₁ subunit in the presence of LPS completely inhibited IL-12p70 secretion compared with IFNAR₂ blocking antibody (Figure. 11.5C). IFN β can bind to IFNAR₁ independently of IFNAR₂ and induce the expression of genes independently of the Jak-STAT pathways [121]. Thus, when IFNAR₂ is blocked, IFN β can still induce gene expression through an IFNAR₁ signaling pathway leading to IL-12p70 secretion. However, blocking of the IFNAR₁ subunit inhibits IFN β signaling and IL-12p70 production. This result confirmed that IFN β signaling through IFN type I receptor is crucial for IL-12p70 secretion.

We next investigated by intracellular flow cytometry which immune cells secrete IFN β in whole blood of six healthy donors after TLR₄ activation. For all donors, we observed IFN β secretion by CD14⁺ monocytes upon LPS stimulation compared with the null condition for which no IFN β secretion was detected (Figure. 11.5D and E). Because of the high IFN β staining levels of unstimulated neutrophils and pDCs we wanted to confirm the LPS monocyte phenotype with another technique. To do this, five cell populations including CD14⁺ monocytes were purified after 3 hours of whole blood stimulation. IFN β was measured by Simoa in cell lysates. Again, IFN β secretion was detected in CD14⁺ monocytes after LPS stimulation compared to the unstimulated control (Figure. 11.5F). To provide insight into variability of the IFN β response, we performed a protein quantitative trait loci analysis after 4 hours LPS stimulation in MI donors. We found significant associations between four genetic variants rs10757182, rs10811473, rs13291085 and rs7020908 and IFN β levels ($P < 4.32e-6$) (Figure. 11.5G). These SNPs have been shown to be in linkage disequilibrium and are located within 1 Mb of the *IFNB1* gene coding for IFN β cytokine. For all SNPs, no significant differences were observed regarding the proportion of IL-12p70 low responders between the 3 allelic dosages of each variant. Interestingly, these variants did not impact the *IFNB1* gene expression as measured by Nanostring (Figure. 11.5B). Overall, we found that the secretion of IFN β by monocytes is crucial to induce IL-12p70 production and, as a consequence the Th1 response upon bacterial infection. In addition, the production of IFN β , but not gene expression, is associated with four cis-acting pQTLs.

11.5. The secretion of IFN β by monocytes is essential for IL-12p70 secretion/Th1 response upon LPS stimulation

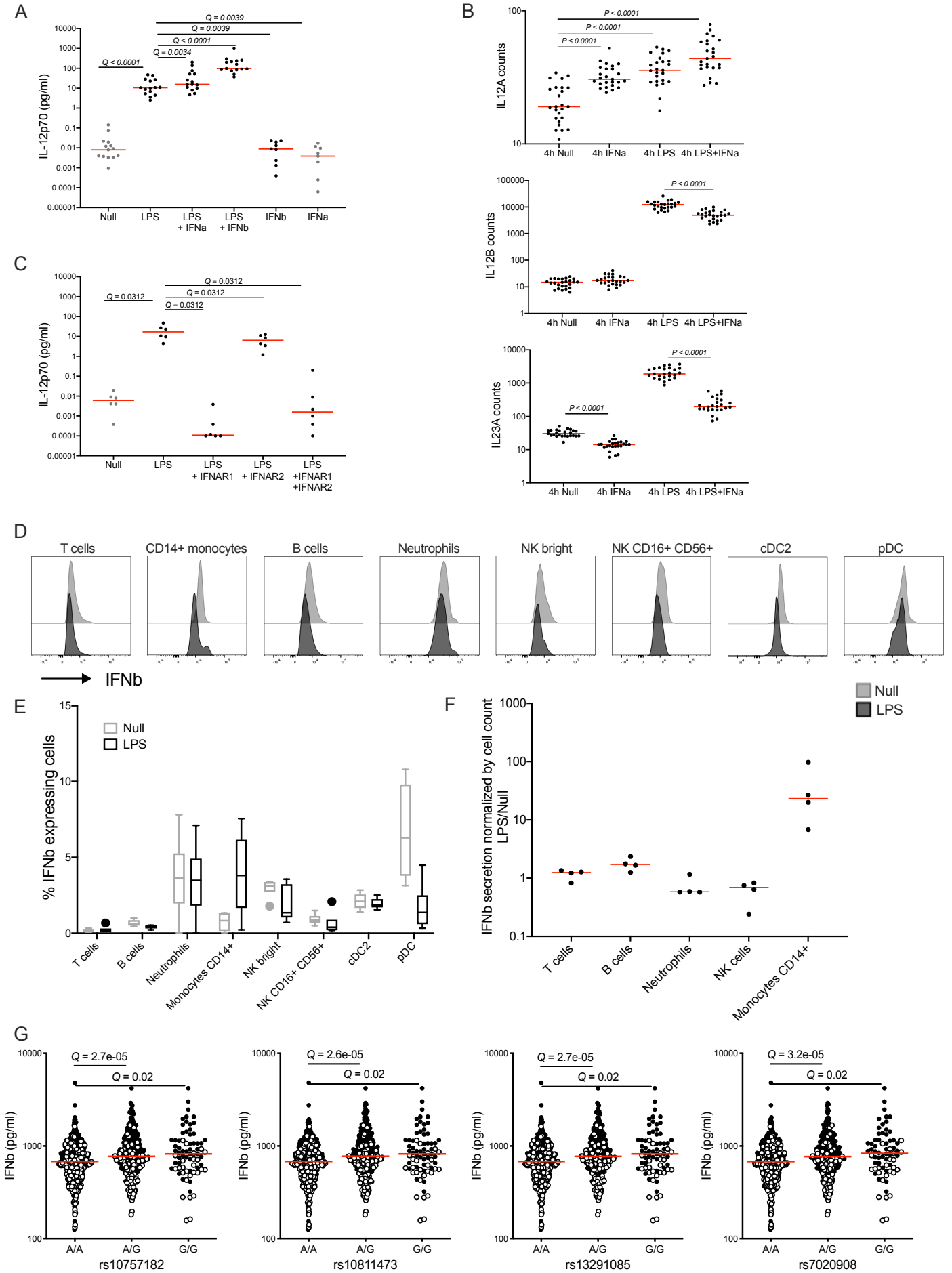


Figure 11.5 (previous page): **Impact of LPS, type I IFNs and IFNAR inhibitors on IL-12p70 production.** (A) IL-12p70 secretion measured by Simoa in whole blood after 22 hours stimulation with LPS, type I IFNs, the combination of LPS and type I IFN (n=9) and an unstimulated control (null). The red lines indicate the median value for each stimulation condition and Q values were determined using the Wilcoxon test and false discovery rate correction was performed to correct for multiple comparison testing. (B) *IL12A*, *IL12B* and *IL23A* gene expression upon 4 hours whole-blood stimulation with IFN α , LPS, LPS+IFN α and an unstimulated control (null), n=25. The red lines indicate the median value for each stimulation condition and P values were determined by the Wilcoxon test. (C) IL-12p70 secretion measured by Simoa in whole blood after 22 hours stimulation with LPS, LPS with IFNAR neutralizing antibodies and an unstimulated control (null). The red lines indicate the median value for each stimulation condition and Q values were determined using the Wilcoxon test and false discovery rate correction was performed to correct for multiple comparison testing. (D) Representative histograms showing intracellular IFN β production measured by flow cytometry in T cells, CD14⁺ Monocytes, B cells, Neutrophils, NK bright cells, NK cells, cDC2 and pDC cell populations after 6 hours of null or LPS whole blood stimulation. (E) Tukey box-whisker plots of the percentage of IFN β cytokine positive cells for the indicated immune cell populations in null and LPS conditions, n=6. (F) Intra-cellular IFN β (LPS/null ratio) measured by Simoa after 3h 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. (G) Cis-acting variants significantly associated with LPS-induced IFN β protein secretion measured in the 1,000 *Milieu Interieur* donors after 22h LPS stimulation.

11.6 *IKBKB* and *IRF5* gene methylation impact the IL-12p70 phenotype

Next, we assessed whether epigenetics may contribute to the IL-12p70 secretion variability utilizing gene methylation data sets from MI donors. To focus our analysis, we only selected genes implicated in the LPS response pathway which totaled 49 genes (listed in Supp Table 1). Of these selected genes we identified 29 probes (out of 1234 probes) corresponding to 20 unique genes (Supp Table 2), that showed significantly different methylation levels between the low responders and the responders (FDR < 0.05). Interestingly, 7 probes showing increased methylation in the responder group were associated to the *IKBKB* and *IRF5* genes (Figure. 11.6A). The *IKBKB* gene codes for the IKKb kinase which phosphorylates and activates the transcription factor IRF5 [234] which plays a role in inducing IFN β expression [235, 236]. 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 condition (Figure. 11.6B). This result highlights the impact of methylation on *IKBKB* and *IRF5* gene expression for downstream cytokine responses to LPS.

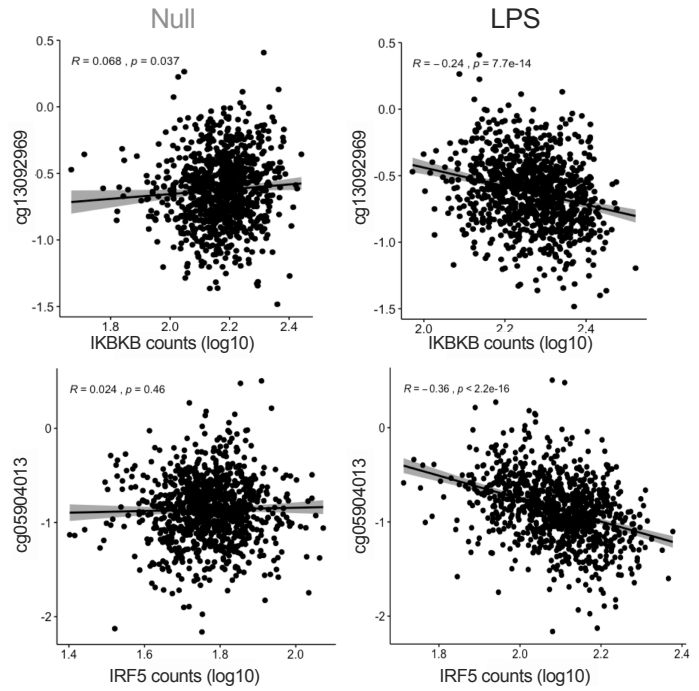
11.7 Integrative model to dissect respective contributions of variable IL12p70 and IFN β responses

To assess the relative importance of intrinsic and genetic factors identified in the different analysis we utilized the feature selection algorithm Boruta [237]. Based on previous studies highlighting the importance of age, sex, smoking, and CMV status to immune variability, these variables were also included in the model. While CMV status and smoking variables showed no importance for both IFN β and IL-12p70 cytokines, sex appeared to have importance for IFN β but not IL-12p70 secretion (Figure. 11.6C). IFN β secretion, genetic variants and HLA-DR MFI in monocytes were the top 3 variables showing high importance for variable IL-12p70 secretion whereas HLA-DR MFI in monocytes and age were particularly important for IFN β secretion. Selected features from the algorithm were integrated in a linear regression model to predict and explain the relationship between IFN β or IL-12p70 secretion and the genetic and nongenetic independent variables (Figure. 11.6D). Age, monocyte HLA-DR expression levels, and genetic variants showed the most significant association with IFN β secretion ($P < 0.001$). HLA-DR MFI in monocytes was also significantly linked with IL-12p70 production in addition to IFN β and genetic variants ($P < 0.001$) confirming our previous analysis. Finally, we quantified the contribution of intrinsic and genetic factors on IFN β and IL-12p70 response variation (Figure. 11.6E). We found that the expression level of cellular activation markers on monocytes and cDC2 had the strongest impact on IFN β secretion explaining 4.4% of the variance, whereas sex explained only 1.1% of the variance. The impact of IFN β production was particularly strong on IL-12p70 secretion as it accounted for 21.6% of the total variance whereas genetics, epigenetics, and cellular markers accounted for less than 5% of the variance individually. In addition, age had a weak impact on IL-12p70 secretion. This analysis also revealed that 67% of variance in IL-12p70 and 88% of variance in IFN β responses remains unexplained in these models.

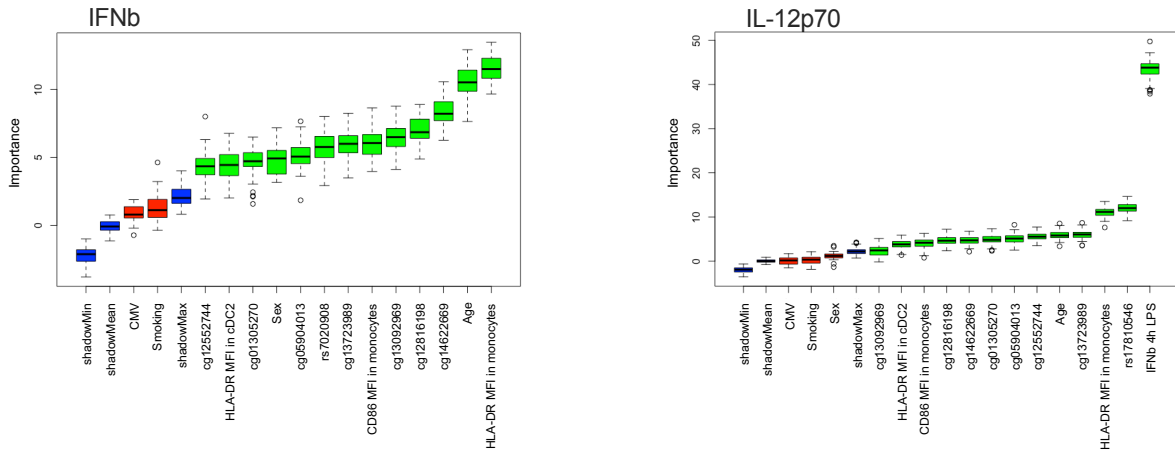
A

Probe	Gene	Q value	Highest methylation group
cg12816198	IRF5	0,016	responders
cg12552744	IKBKB	0,016	responders
cg13723989	IKBKB	0,021	responders
cg01305270	IKBKB	0,021	responders
cg05904013	IRF5	0,025	responders
cg14622669	IRF5	0,035	responders
cg13092969	IKBKB	0,039	responders

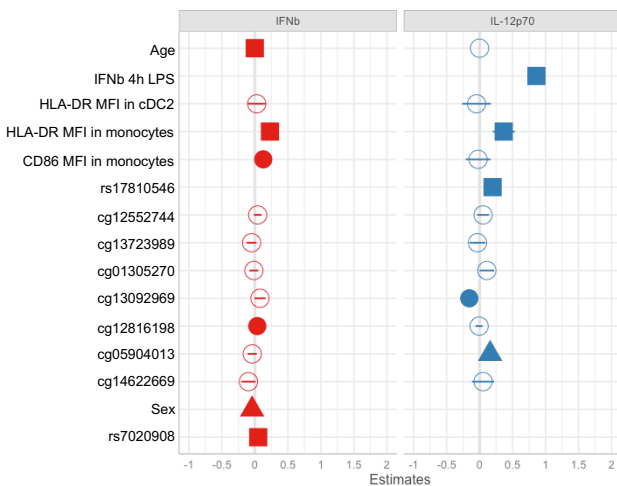
B



C



D



E

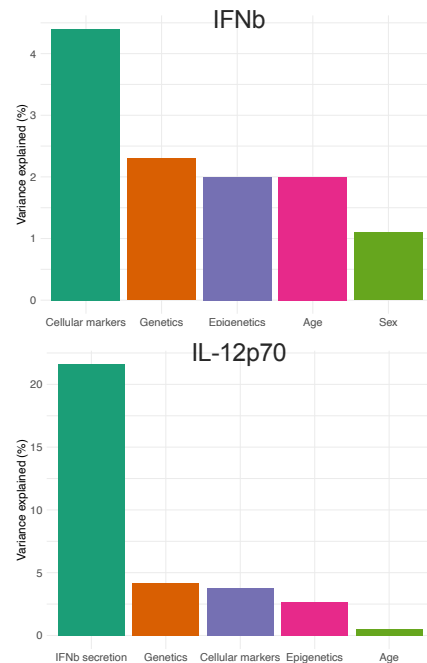


Figure 11.6 (previous page): **Host genetics and non-heritable factors influence IFN β and IL-12p70 secretion.** (A) List of *IKBKB* and *IRF5* gene methylation probes significantly different between the *Milieu Interieur* low responders and responders. (B) Correlation of one *IKBKB* and *IRF5* probe methylation values with their respective gene expression measured by Nanostring after null and LPS 22h stimulations. Pearson correlations were performed using gene expression log₁₀ values. (C) IFN β 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 boxplots represent Z scores of rejected and confirmed attributes respectively. (D) Forest plots displaying the statistical significance of each variable associated with IFN β and IL-12p70 resulting from the predictive linear regression models. (E) Proportion of the variance explained by genetic and nongenetic factors regarding IFN β and IL-12p70 secretion following whole-blood LPS stimulation at 4h and 22h, respectively.

11.8 TLR₄ – IFN β – IL-12p70 pathway is altered in HCV-infected individuals who are clinical non-responders to interferon therapy

To determine whether the IL-12p70 response is also associated with IFN β secretion in disease settings, we examined secretion of type I IFNs and IL-12p70 in TB and HCV patients. These two diseases were chosen to have both bacterial and viral infections, and because IFN gene signatures have been implicated in chronic infections. Furthermore, the HCV patients were treated with 6 months of pegylated interferon plus ribavirin, which was the standard of care treatment at the time of the clinical study. This provided the opportunity to test the relevance of our findings in a relevant therapeutic setting. Patient cohorts were established as described in the methods and whole blood was stimulated for 22 hours with LPS and an unstimulated control (null) using the same TruCulture system as applied to MI donors. For tuberculosis infection no significant difference was observed between latently infected controls (LTBI) and active TB patients regarding type I IFNs and IL-12p70 secretion after LPS stimulation (Figure. 11.7A). In both latent *M.tb* infection and active TB disease, IL-12p70 secretion was positively and significantly correlated with IFN β cytokine secretion (Pearson's R value 0.76 and 0.86 respectively, $P < 0.0001$) (Figure. 11.7B and C), showing that the IFN β IL-12p70 pathway is not perturbed in the latent or active TB individuals. Moreover, IL-12p70 secretion was also correlated with IFN α secretion (Pearson's R value 0.67 and 0.62, $P < 0.001$) but to a lesser extent confirming the strong link between IFN β and IL-12p70 secretion in TB disease setting. For HCV infection, blood was stimulated with both LPS and a null control, after which we measured IFN β and IL-12p70 responses by Simoa. Patients were divided into those

that were sustained virological responders (SVR+) or not (SVR-) after 6 months of treatment with pegylated interferon plus ribavirin. No significant differences were observed in IL-12p70 and IFN β secretion between the SVR+ and SVR- patients for any of the stimulation conditions (Figure. 11.7D). However, IFN β 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. 11.7E). These results show that the IFN β -IL-12p70 pathway is altered in a subgroup of HCV infected patients who do not respond clinically to IFN-based treatment.

11.8. TLR4 – IFN β – IL-12p70 pathway is altered in HCV-infected individuals who are clinical non-responders to interferon therapy

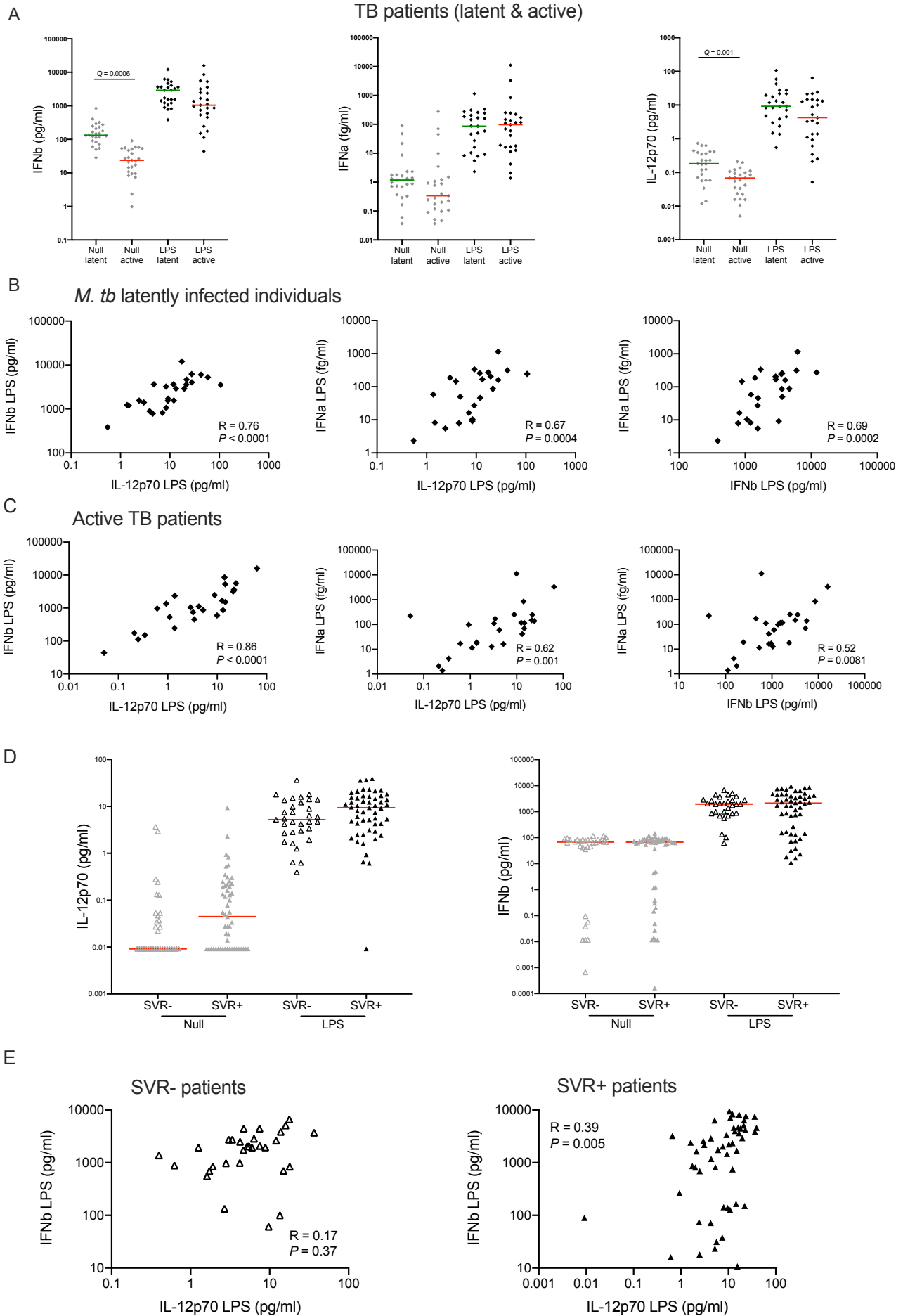


Figure 11.7 (previous page): **IL-12p70 response is associated with IFN β secretion in bacterial and viral infections.** (A) Type I IFNs and IL-12p70 secretion measured by Simoa after 22h null and LPS stimulation of whole blood in *M. tb* latently infected individuals and active TB patients. The red lines indicate the median value for each stimulation and group condition. Q values were determined by the Mann-Whitney test and false discovery rate corrected for multiple comparison testing, n=25 donors per group. (B)(C) Correlation between IFN α , IFN β or IL-12p70 secretion measured by Simoa after 22 hours of LPS stimulation in latent *M. tb* individuals (B) and active TB patients (C). Pearson correlation tests were performed using log₁₀ values. (D) IL-12p70 and IFN β secretion measured by Simoa after 22h null and LPS stimulation of whole blood in SVR+ (n=54) and SVR- (n=33) HCV infected patients defined after 6 months of IFN α -based therapy. The red lines indicate the median value for each stimulation and group condition. P values were determined by the unpaired t test. (E) Correlation between IFN β 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.

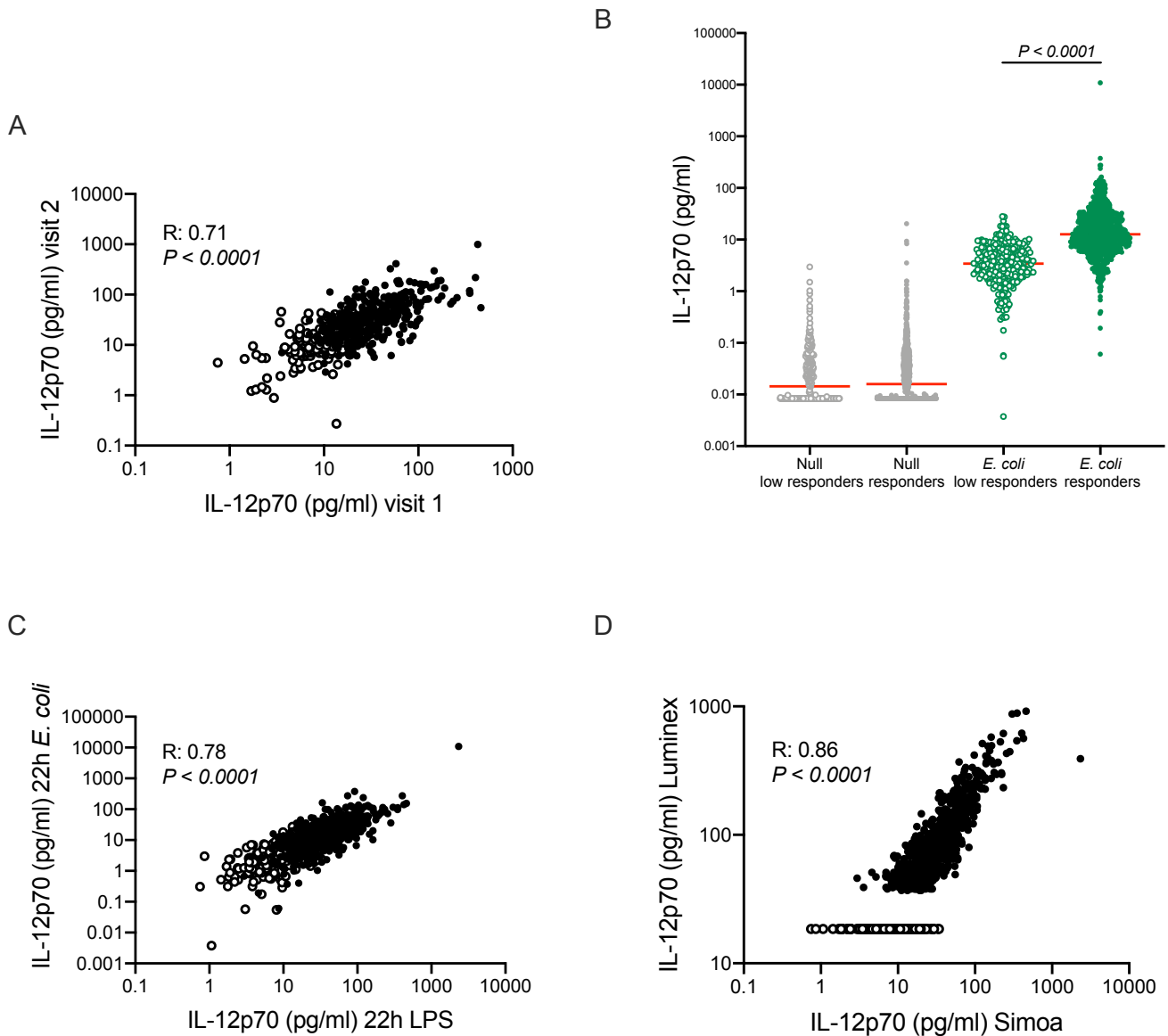


Figure 11.S1: **Supplementary Figure 1.** (A) Correlation of IL-12p70 secretion after LPS 22h stimulation at 2 separate time points. Pearson correlation was performed on log₁₀ values. (B) IL-12p70 secretion measured by Simoa in the two groups of *Milieu Interieur* IL-12p70 responders after null and *E. 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. (C) Correlation of IL-12p70 secretion measured by Simoa after *E. coli* and LPS 22h stimulation. Pearson correlation was performed using log₁₀ values. (D) Correlation of IL-12p70 secretion after LPS 22h stimulation measured by Luminex and Simoa. Pearson correlation was performed using log₁₀ values. In each plot, empty circles represent IL-12p70 low responders of the *Milieu Interieur* cohort.

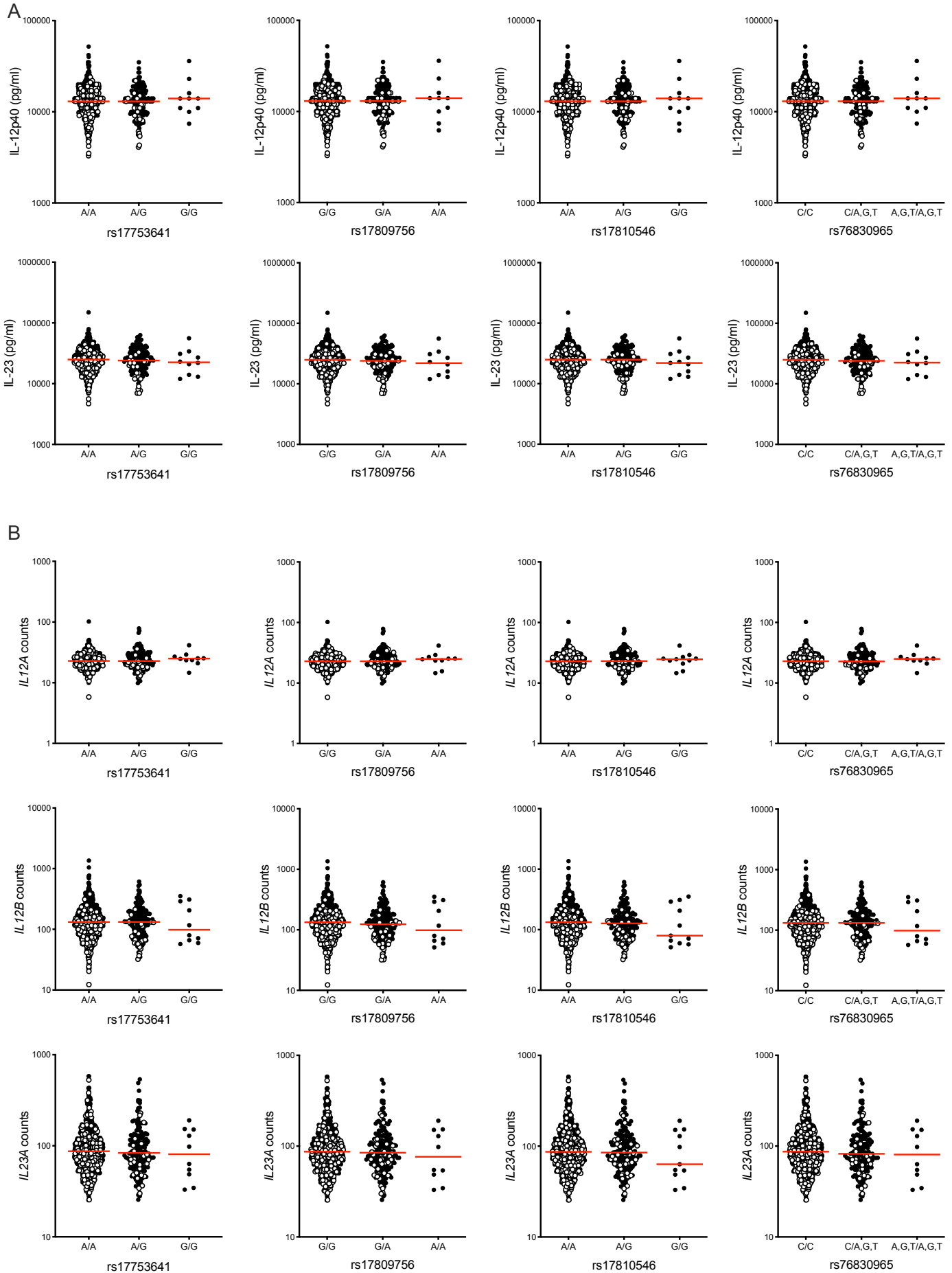


Figure 11.S3 (previous page): **Supplementary Figure 3.** (A) IL-12p40 and IL-23 protein secretion in the 1,000 *Milieu Interieur* individuals following 22 hours LPS stimulation according to the rs17753641, rs17809756, rs17810546 and rs76830965 genotypes. (B) *IL12A*, *IL12B*, and *IL23A* gene expression measured by Nanostring after 22 hours LPS stimulation according to the rs17753641, rs17809756, rs17810546 and rs76830965 genotypes. 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 (on log₁₀ values) and false discovery rate correction was performed to correct for multiple comparison testing.

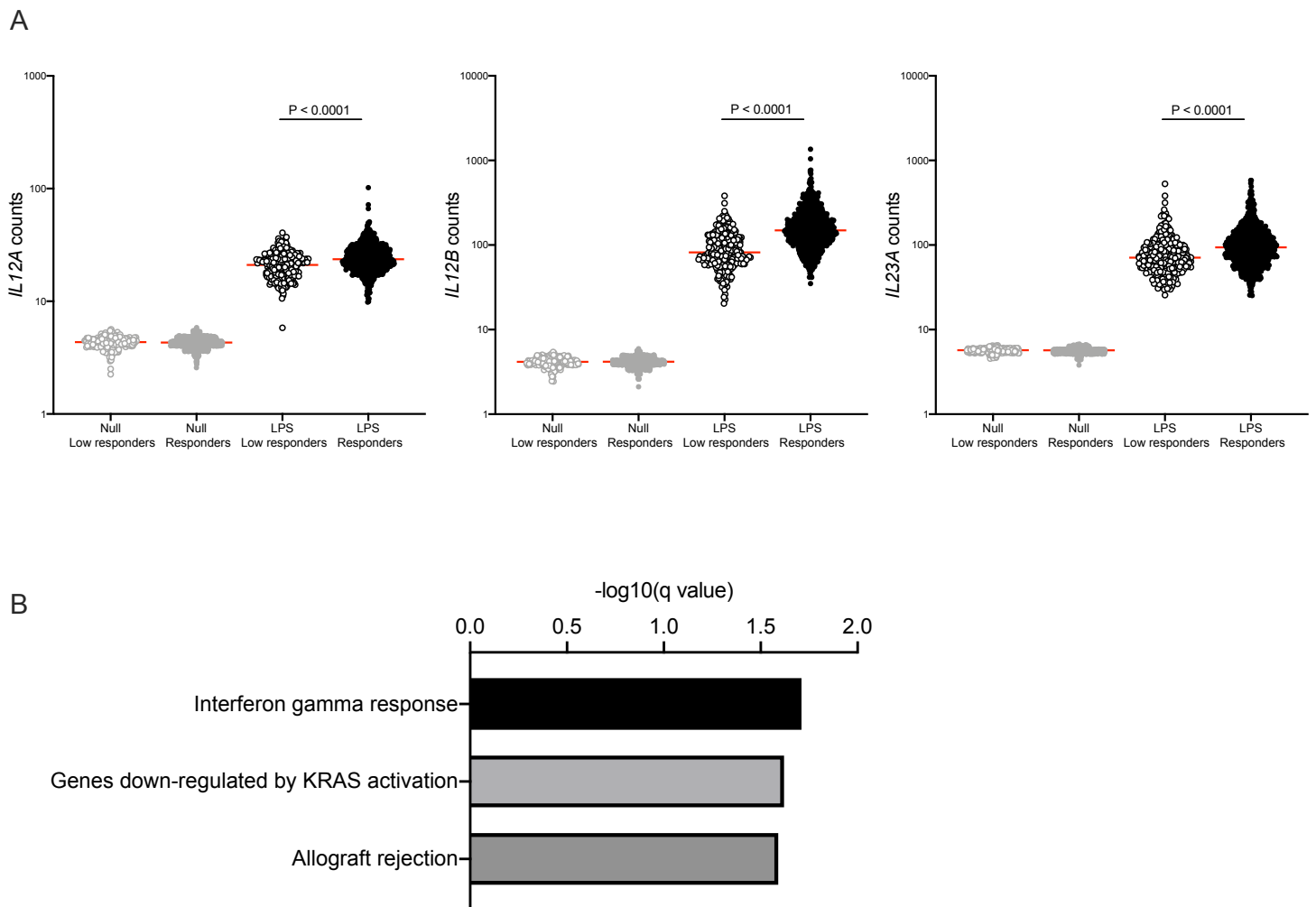


Figure 11.S4: **Supplementary Figure 4.** (A) *IL12A*, *IL12B* and *IL23A* gene expression measured by Nanostring in the two groups of *Milieu Interieur* IL-12p70 responders after null and LPS stimulation. Red lines indicate the median value for each group. P values were determined by an unpaired Student's t test on log₁₀ values. (B) Gene set enrichment analysis bar plot displaying the significant pathways from the comparative analysis between the low responders and the responders following LPS 22 hours stimulation using the Molecular Signatures Database Hallmark gene sets.

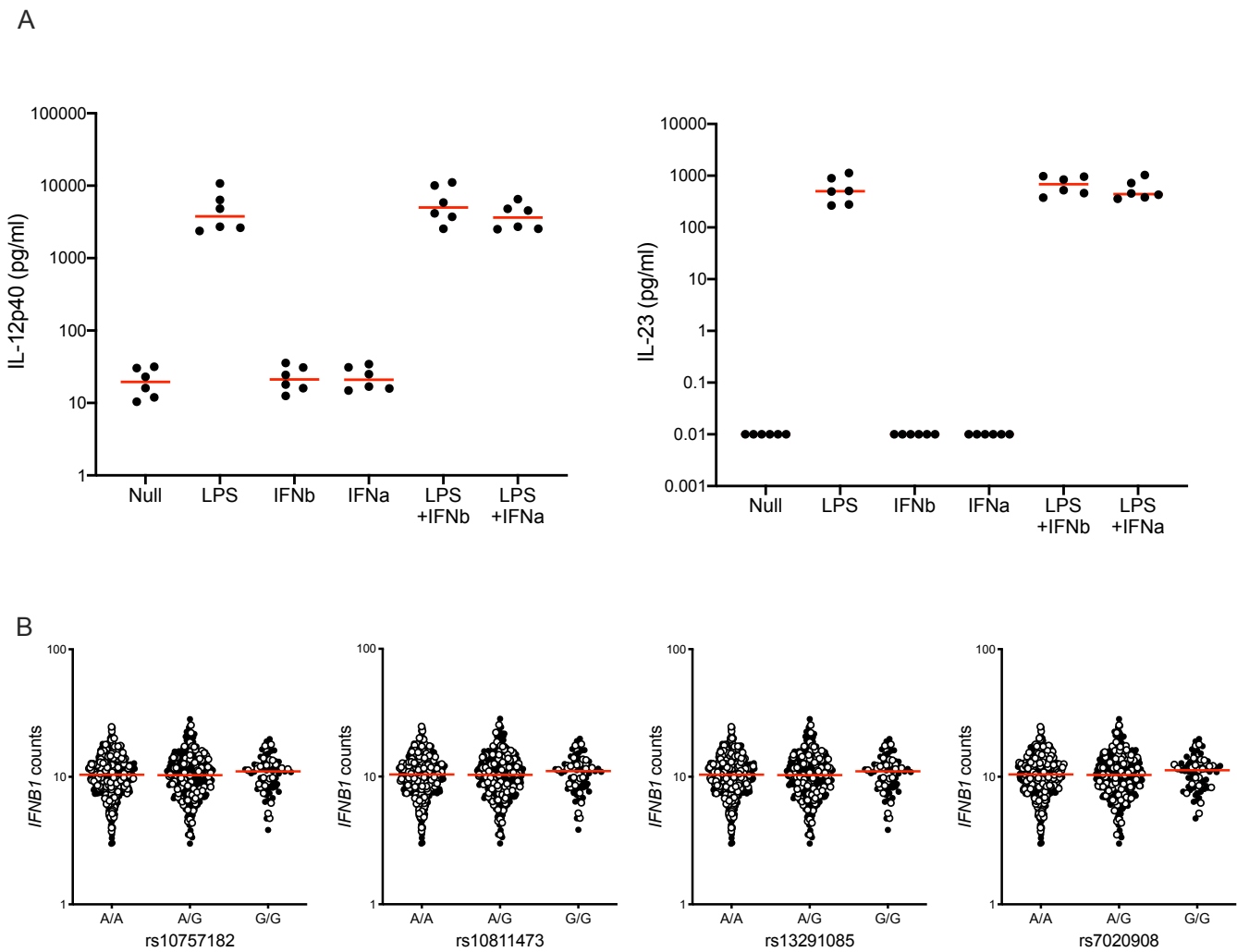


Figure II.S5: **Supplementary Figure 5.** (A) IL-12p40 and IL-23 protein secretion measured by Simoa in whole blood after 22 hours stimulation with LPS, type I IFNs, the combination of LPS and type I IFN and an unstimulated control (null), $n=6$. The red lines indicate the median value for each stimulation condition. (B) Dot plots show rs10757182, rs10811473, rs13291085 and rs7020908 genotype-stratified gene expression levels for the *IFNB1* gene measured by Nanostring after 22h 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 (on log₁₀ values) and false discovery rate correction was performed to correct for multiple comparison testing.

LPS pathway genes	
CD14	REL
LBP	JUN
TLR4	TRAM1
LY96	TICAM1
TIRAP	RIPK1
MYD88	TRAF3
IRAK4	TANK
TRAF6	TBK1
MAP3K7	IKBKE
MAP2K1	IRF1
MAP2K2	IRF2
MAP2K3	IRF3
MAP2K4	IRF4
MAP2K6	IRF5
MAP2K7	IRF6
MAPK14	IRF7
MAPK11	IRF8
MAPK8	IRF9
MAPK3	UBE2V1
MAPK1	UBE2N
IKBKB	NFKB1
CHUK	NFKB2
NFKBIA	RELA
NFKBIL1	RELB
IFNB1	

Table 11.1: **Supplementary Table 1.** LPS pathway genes used for the differential methylation level analysis.

Probe	Gene	Q value
cg12816198	IRF5	0,016
cg12552744	IKBKB	0,016
cg21045241	RELB	0,021
cg01037764	TRAM1	0,021
cg13723989	IKBKB	0,021
cg01305270	IKBKB	0,021
cg21053741	NFKBIL1	0,021
cg16604658	TBK1	0,025
cg04666274	MAP2K2	0,025
cg05904013	IRF5	0,025
cg06392169	IRF4	0,025
cg19308985	IRF8	0,035
cg20567119	TRAF6	0,035
cg16289653	MAP2K2	0,035
cg11917175	IRF2	0,035
cg14622669	IRF5	0,035
cg14456745	TANK	0,035
cg16145324	MAPK14	0,035
cg24064264	IRF7	0,035
cg13730105	TLR4	0,037
cg10080401	MAPK1	0,037
cg04639174	TRAF6	0,037
cg13092969	IKBKB	0,039
cg04123016	MAP2K1	0,041
cg09991769	TRAF3	0,043
cg12323063	MAP2K6	0,043
cg23044504	NFKB2	0,044
cg22819055	TRAF3	0,044
cg12970512	TRAF3	0,046

Table II.2: **Supplementary Table 2.** Probes showing significantly different methylation levels between the low responders and the responders.

12. Additional hypothesis tested

12.1 Role of type II IFN in IL-12p70 secretion

Although it is well described in the literature that IL-12p70 induces IFN γ [26, 238], some studies have shown that a first wave of IFN γ is necessary to induce IL-12p70 [231, 232, 233]. IFN γ priming before LPS stimulation upregulates both p35 (*IL12A*) and p40 (*IL12B*) gene expression [231] and IFN γ induced by IL-12p70 further enhances IL-12p70 creating a positive feedback loop [50]. These findings highlight the importance of IFN γ in IL-12p70 secretion. Moreover, Kraaij et al. observed that human monocytes can produce IFN γ upon stimulation with LPS [239]. Based on these papers, we hypothesized that monocytes and DCs can produce IFN γ following whole-blood LPS stimulation and that the low responders of the MI cohort might have a defect in IFN γ secretion that could be responsible for low IL-12p70 production. To test this hypothesis, we performed three different experiments to (i) validate that monocytes and DCs secrete IFN γ upon LPS whole-blood stimulation, (ii) measure early IFN γ secretion in some low responders and responders of the MI cohort to examine whether there is any significant difference between the two groups and (iii) assess the kinetics of IFN γ and IL-12p70 using the ultrasensitive Simoa technology.

12.1.1 Assessing IFN γ secretion by monocytes and DCs following LPS stimulation

To determine whether monocytes and DCs are able to secrete IFN γ as reported by Kraaij et al. [239], the blood of 9 healthy donors was stimulated for 22 hours with Null and LPS in the presence of brefeldin A 8 hours after the beginning of the stimulation. Intracellular staining for IFN γ cytokine was performed by flow cytometry. Although a clear IFN γ secretion was observed in T cells and NK cells after 22h LPS stimulation, we did not observe IFN γ secretion in CD14⁺ monocytes and cDC2 (Figure. 12.1). To test whether IFN γ secretion occurs in monocytes and DCs before 8 hours of stimulation, the blood of 3 healthy donors was stimulated with Null and LPS in the presence of brefeldin A for 8h and IFN γ secretion was studied by flow cytometry. Again, we could not detect IFN γ

in monocytes and DCs of these 3 donors (data not shown).

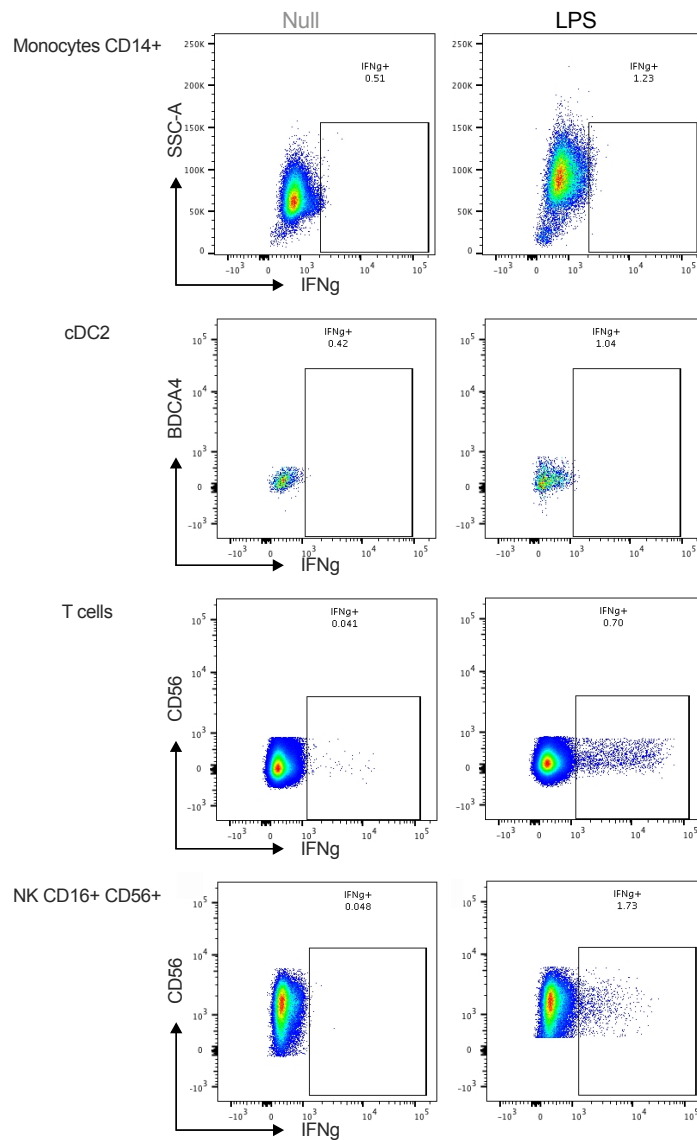


Figure 12.1: **IFN γ secretion in monocytes, cDC2, T cells and NK cells upon Null and LPS stimulations.** Whole-blood from 9 healthy donors was stimulated for 22 hours with no stimulus (Null) and LPS in the presence of brefeldin A (added 8 hours after the stimulus) and assessed for IFN γ cytokine production using flow cytometry in different immune cell types. After isolating the immune cell populations of interest, the limit of positivity for IFN γ secretion was defined using the Null condition and applied to the LPS condition in order to see if LPS induces IFN γ secretion in these immune cells.

12.1.2 Early IFN γ secretion in *Milieu Interieur* low responders and responders

Early protein secretion differences observed between the two groups after 4 hours of stimulation may contribute to explain the IL-12p70 low responder phenotype observed after 22 hours of stimulation. To define whether the low responders secrete less IFN γ than the responders at an early timepoint of stimulation, we measured IFN γ by Simoa in Null and LPS TruCulture supernatants of 50 low responders and 50 responders after 4 hours of stimulation. The 100 selected donors were all part of the 20-39 years old group to avoid age impact on data analysis. IFN γ was not significantly more secreted in the supernatant of the responders after 4 hours LPS stimulation compared with the low responders (FDR threshold of 0.01) (Figure. 12.2). The difference was more important between the two groups in the Null 4h stimulation condition with a higher baseline level of IFN γ in the responders that could potentially help priming and induce IL-12p70 [240].

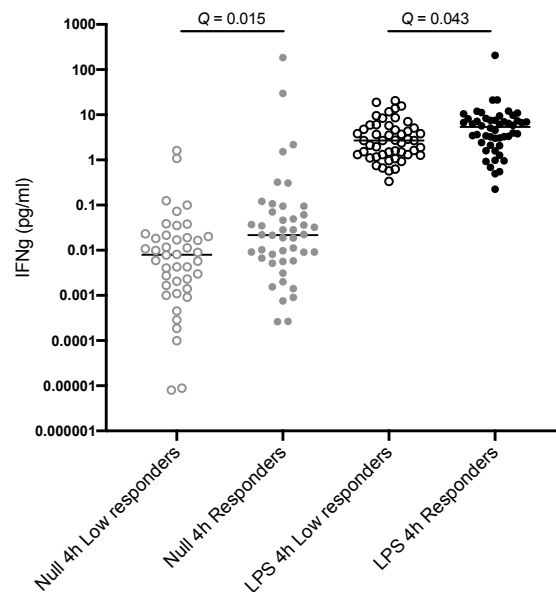


Figure 12.2: IFN γ levels in 100 *Milieu Interieur* donors after 4h of Null and LPS stimulations. IFN γ cytokine concentration was measured in Null and LPS TruCulture supernatants of 100 MI healthy donors (half responders and half low responders) using an IFN γ Simoa homebrew assay. Unpaired t tests on logged values were performed between the two groups for Null and LPS stimulations and p values were corrected using the FDR method.

12.1.3 Kinetic of IFN γ and IL-12p70 secretion using ultrasensitive Simoa assays

To define whether IFN γ is produced upstream or downstream of IL-12p70, the blood of five healthy donors was stimulated over time at 14 different timepoints from 0 to 30 hours with LPS. IL-12p70 and IFN γ were measured using Simoa assays. Examining the data of these two assays, we can observe that in 3 out of 5 donors, IFN γ is clearly induced after IL-12p70 secretion under LPS stimulation (Figure. 12.3). In donor 2, the production of the two cytokines was simultaneous whereas no clear result was obtained for donor 3. Thus, in 4 out of 5 donors, IFN γ was not secreted upstream of IL-12p70 suggesting that IL-12p70 induces IFN γ as reported in the literature [26, 238] and that IFN γ cannot be responsible for less IL-12p70 secretion in the low responder group of healthy donors.

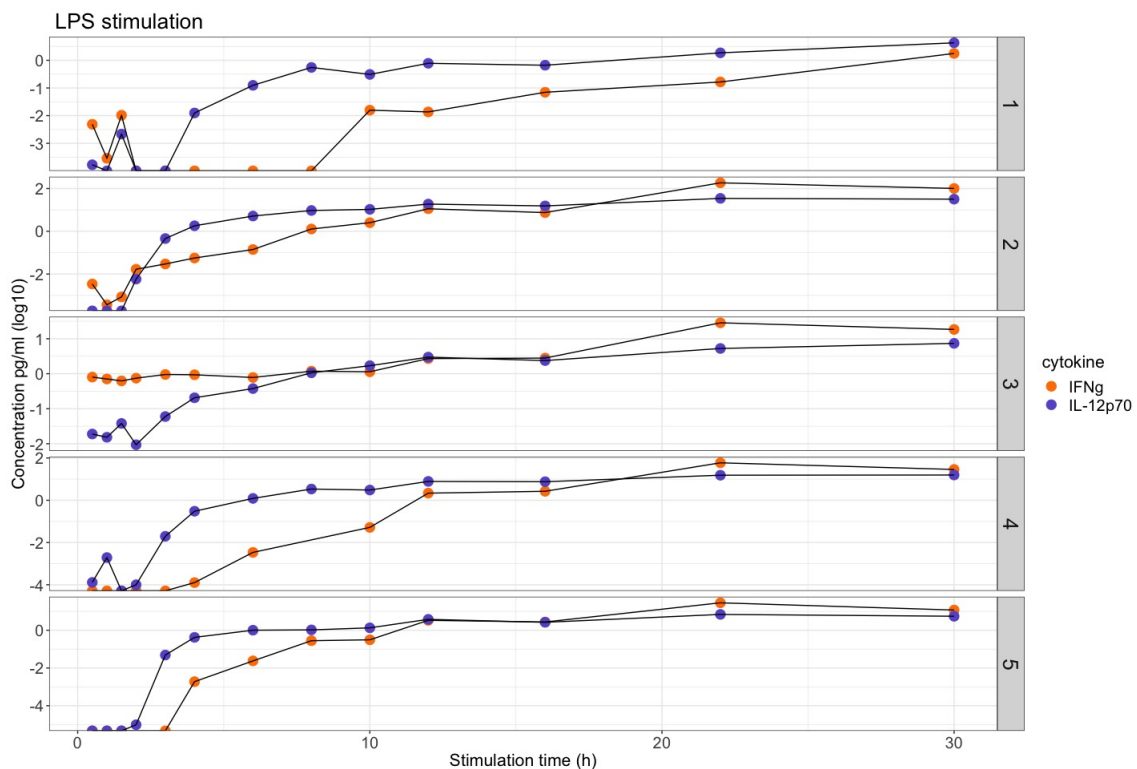


Figure 12.3: IFN γ and IL-12p70 secretion over time upon LPS stimulation in five healthy donors. Whole blood of five healthy donors was stimulated in LPS TruCulture tubes at 14 different timepoints from 0 to 30 hours. IFN γ and IL-12p70 were measured in the supernatant using Simoa homebrew assays.

12.1.4 Conclusions

In summary, we could not confirm that monocytes and DCs secrete IFN γ upon LPS stimulation by flow cytometry as described in the literature [239]. Secondly, we did not observe a highly significant difference in IFN γ secretion between the responders and the low responders after 4 hours of LPS stimulation. Finally, when measuring IL-12p70 and IFN γ secretion over time in five healthy donors, we showed that IL-12p70 is produced upstream of IFN γ . Collectively, these results do not support our hypothesis that IFN γ is a potential upstream regulator of IL-12p70 cytokine variability following LPS stimulation.

12.2 Unbiased transcriptomic analysis

The Nanostring Human Immunology v2 gene expression panel allows the analysis of a restricted number of genes. Thus, we decided to apply an RNA-sequencing (RNA-seq) unbiased approach in 15 low responders and 15 responders to screen a wide number of genes and potentially identify new biomarkers characterizing the phenotype. All selected donors were part of the 20-29 years old group and equally distributed by sex. The stimulated conditions that were sequenced included Null 4 hours, LPS 4 hours and LPS 22 hours. Globin and ribosomal RNA (rRNA) were removed and a minimum of 120 million reads of 101 base-pair (bp) were generated per sample.

12.2.1 Data pre-processing

12.2.1.1 Mapping and count assignment analysis

After performing all quality controls on the raw data files (data not shown), we controlled for rRNA quantity as it has an effect on the usable number of reads. Although both cytoplasmic and mitochondrial rRNA were removed during the library preparation, we observed that around 10% of the reads corresponded to rRNA with no significant differences between the stimulation conditions (Figure. 12.4A). The proportion of uniquely mapped read was around 80% for all stimulation conditions indicating that the quality of the libraries as well as the data processing were good [241] (Figure. 12.4B). In the end, 40 to 60 million reads were assigned to a gene (Figure. 12.4C).

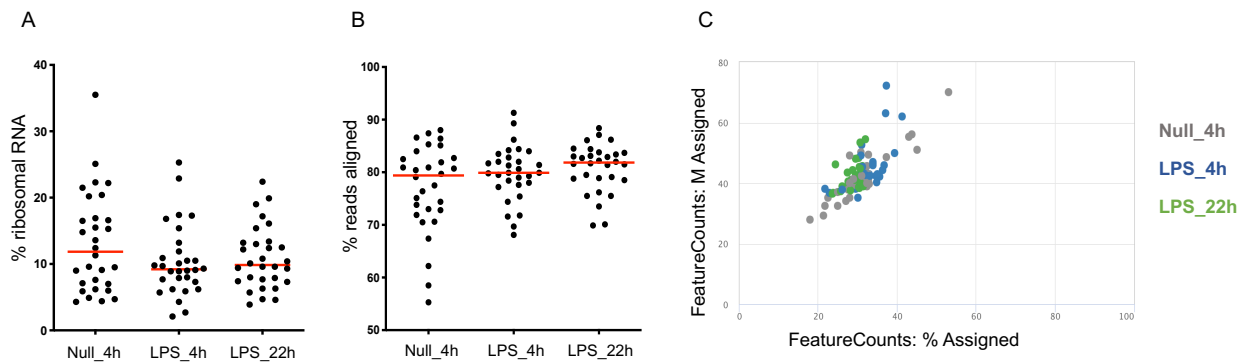


Figure 12.4: **RNA-sequencing alignment and read quantification.** Whole blood RNA of 15 low responders and 15 responders was sequenced. (A) Ribosomal RNA was quantified for each stimulation condition and removed from the analysis. (B) Percentage of reads aligned to the human transcriptome. (C) Number and percentage of assigned counts color-coded by stimulation condition.

12.2.1.2 Experimental variability

When performing RNA-seq, the main variability within the experiment is expected to come from biological differences between the samples which corresponds to the stimulation conditions. A variance stabilizing transformation (VST) was performed to transform the dataset and make the variance independent of the mean generating homoscedastic data. Then, we performed hierarchical clustering to check that samples cluster by biological condition. As expected, the dendrogram grouped the samples by stimulation conditions (Figure. 12.5).

The second way to visualize the experiment variability is to examine the first principal components. We analyzed the data by principal component analysis (PCA). The first three principal components explained 50% of the variability and again, this analysis revealed stimuli-specific clusters confirming that the biological conditions were the main source of variance in the data (Figure. 12.6).

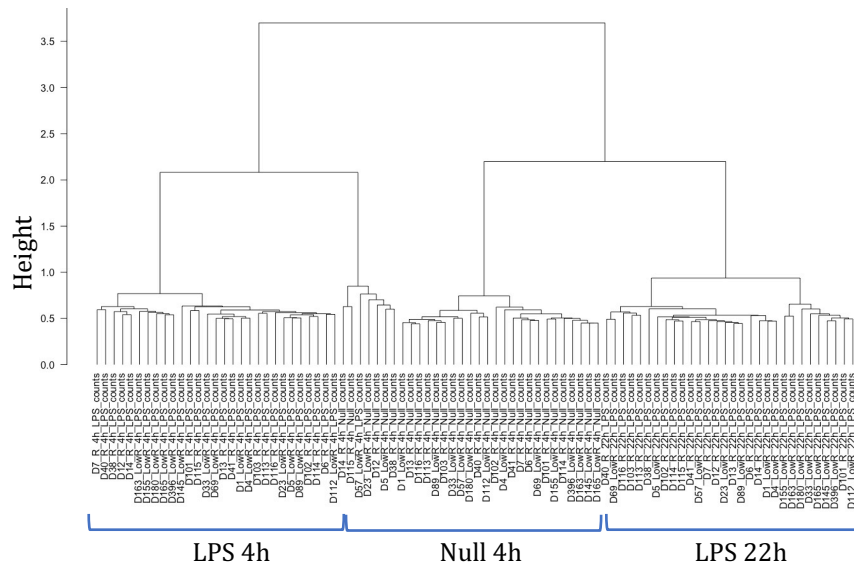


Figure 12.5: **Cluster dendrogram of all sequenced samples.** The Euclidian distance was computed between each sample and the dendrogram was built based on Ward criterion.

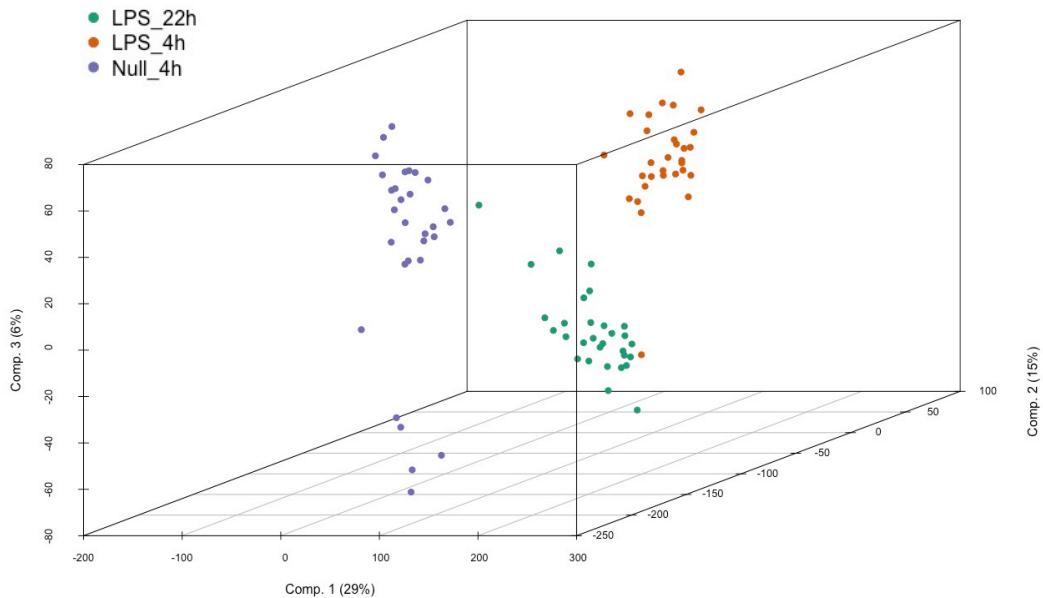


Figure 12.6: **Principal component analysis (PCA) color-coded by stimulation condition.** PCA was performed on the entire RNA-sequencing data set. Plot represents the first three components of the PCA including percentage of variance link to each axis.

12.2.2 Differential gene expression analysis between stimulation conditions

12.2.2.1 Differential gene expression

We performed differential gene expression (DGE) analysis between all stimulation conditions using DESeq2. We then generated MA-plots to have an overview of the results with a two-group comparison (Figure. 12.7). The \log_2 fold change of each comparison was plotted on the y-axis and the average of the counts normalized by the size factor was shown on the x-axis. We observed that 38% of the genes were differentially expressed ($FDR < 0.01$) between the Null 4h and LPS 4h comparisons whereas 20.4% of the genes were differentially expressed between the Null 4h and LPS 22h suggesting that a short stimulation timepoint induces more gene expression differences than a longer stimulation timepoint. In addition, 39% of the genes were differentially expressed in the 4h and 22h LPS two-group comparison indicating again that the stimulation timepoint is crucial to observe gene expression differences.

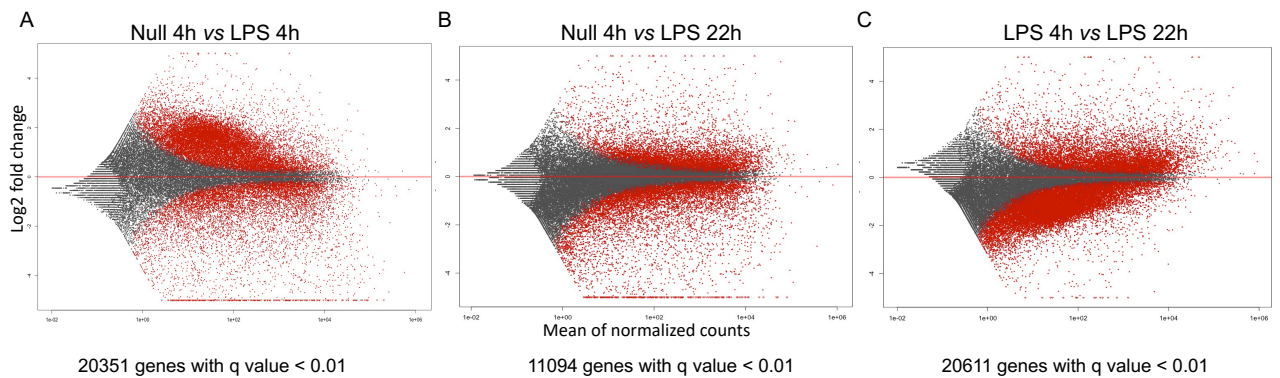


Figure 12.7: **MA-plots comparing all stimulation conditions two by two.** (A) MA-plot of the data for the comparison Null 4h vs LPS 4h. (B) MA-plot of the data for the comparison Null 4h vs LPS 22h. (C) MA-plot of the data for the comparison LPS 4h vs LPS 22h. Each dot represents one gene and red dots represents significantly differentially expressed genes ($FDR < 0.01$).

We also constructed a heatmap of the 30 top differentially expressed genes color coded by sex and stimulation condition using variance-stabilized transformed values and all stimulation conditions clustered separately (Figure. 12.8). However, the males and females were not clustered separately suggesting that sex did not impact the DGE analysis of these genes when comparing the stimulation conditions for these selected genes.

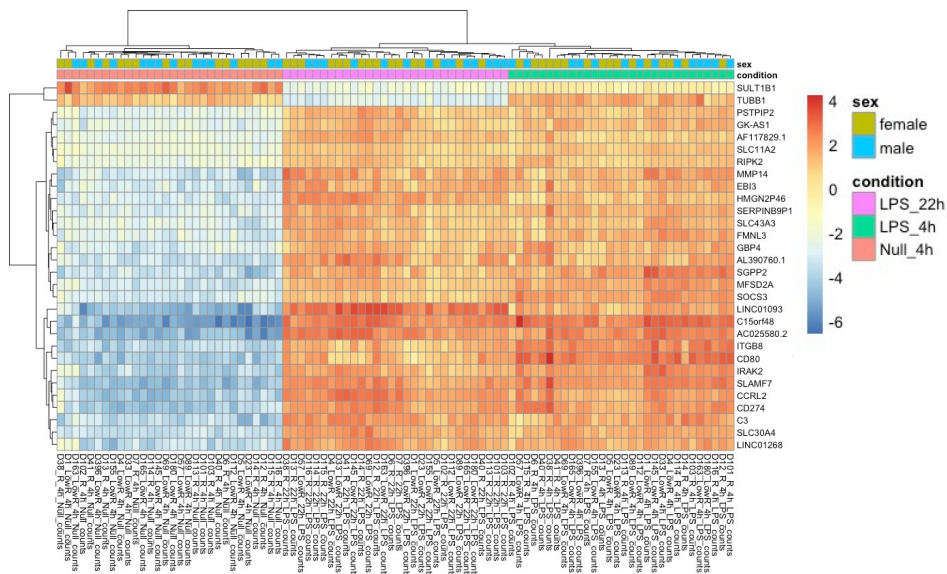


Figure 12.8: **Heatmap of the top 30 differentially expressed genes.** Rows and columns were clustered using hierarchical clustering using variance-stabilized transformed values.

12.2.2.2 Nanostring RNA-sequencing comparison

Next, we compared induced gene expression of Null 4h and LPS 22 hour conditions between RNA-seq and Nanostring. For this, we used the gene expression of 554 genes measured with both technologies. Nanostring data were available for 25 donors and RNA-seq data of the 30 sequenced donors were used. Both technologies displayed a strong positive correlation in the transcriptomic pattern of the comparable genes with a correlation coefficient of 0.93 and a highly significant p value ($P < 2.2 \times 10^{-19}$) (Figure. 12.9). This result demonstrates that both platforms are reliable and generate comparable results.

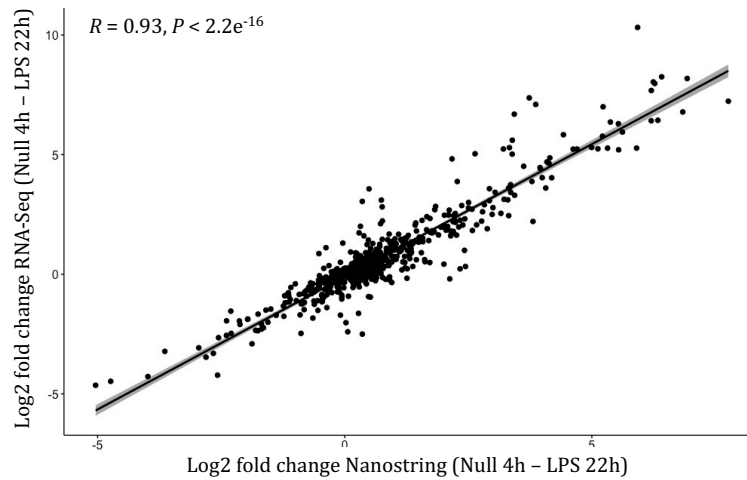


Figure 12.9: **Comparison of RNA-sequencing and Nanostring data.** Correlation plot of the log₂ fold change Null 4h vs LPS 22h conditions between RNA-seq and Nanostring gene expression data.

12.2.2.3 IFN gene expression

We observed the distribution of the normalized counts per stimulation conditions for specific genes of interest, specifically type I IFNs genes (Figure. 12.10). *IFNB1* gene was specifically induced with LPS 4h condition whereas *IFNA2* was induced both following 4h and 22h LPS stimulations. In addition, *IFNB1* gene (100 to 1000 counts) was 10 times more induced than *IFNA2* gene (5 to 15 counts) after LPS 4h stimulation showing the specificity of LPS to induce $IFN\beta$, and supporting our results with protein data. Regarding the gene expression of the type I IFN receptor, the expression of *IFNAR1* was stable between the three analyzed conditions while *IFNAR2* gene expression was downregulated in the LPS 4h condition. This observed downregulation of *IFNAR2* expression following early LPS stimulation suggests a mechanism to reduce type I IFN mediated responses.

Our previous results revealed that $IFN\beta$ drives inter-individual variability in IL-12p70 secretion. As $IFN\beta$ gene expression is induced following 4 hours of LPS stimulation but not 22 hours LPS stimulation, we hypothesized that potential interesting gene expression differences between the low responders and the responders would be observable using the LPS 4h stimulation condition.

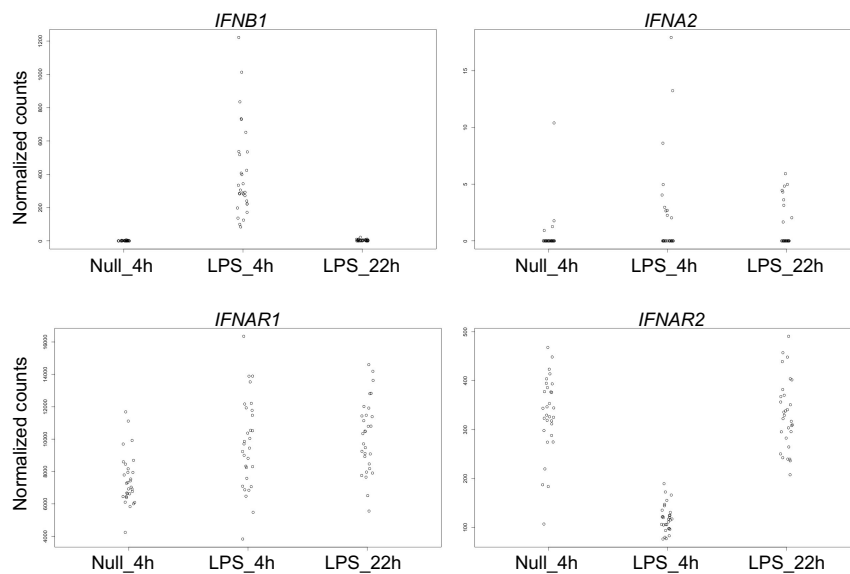


Figure 12.10: **Expression of type I IFNs related genes by stimulation condition.** *IFNB1*, *IFNA2* and type I IFNs receptor gene counts were normalized and plotted according to each stimulation condition.

12.2.3 Differential gene expression analysis between responders and low responders

After performing DGE analysis between stimuli, we assessed whether genes are differentially expressed between the 15 low responders and 15 responders sequenced within each stimulation condition.

12.2.3.1 Null 4 hours stimulation condition

To assess whether the low responders and the responders cluster separately in the 4h Null condition, we performed a PCA color-coded by response (Figure. 12.11A) and sex (Figure. 12.11B). The first two principal components explained more than 60% of the variability. Two distinct clusters are present on the PCA plots which were not associated to the response factor but were explained by the sex factor. Thus, the principal cause of variation in this stimulation condition is the biological factor sex rather than the IL-12p70 response.

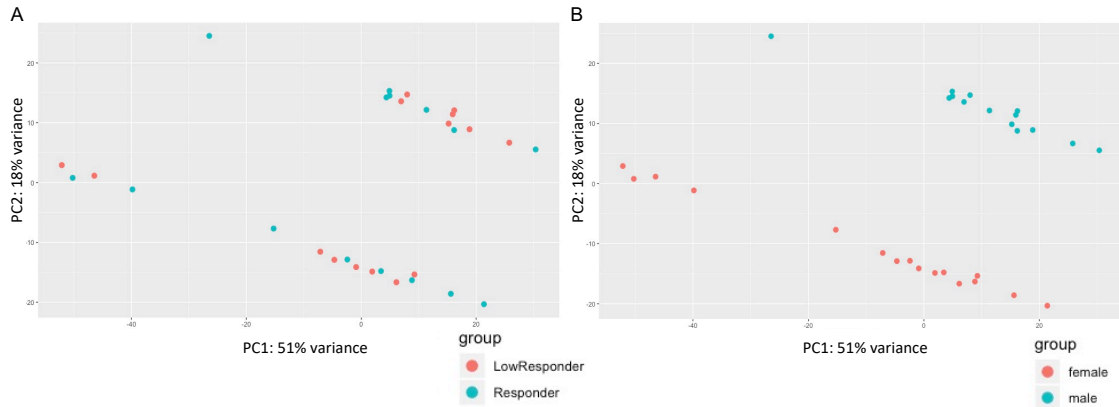


Figure 12.11: **Null 4 hours condition principal component analysis (PCA) color-coded by IL-12p70 response (A) and sex (B).** Plots represent the first two components of the PCA including percentage of variance link to each axis.

We next performed DGE analysis between the two groups and visualized the results in a MA-plot (Figure. 12.12). No genes were significantly differentially expressed between the two groups out of more than 50,000 genes studied suggesting that the basal gene expression levels do not explain the observed phenotype in these small number of donors (FDR < 0.01).

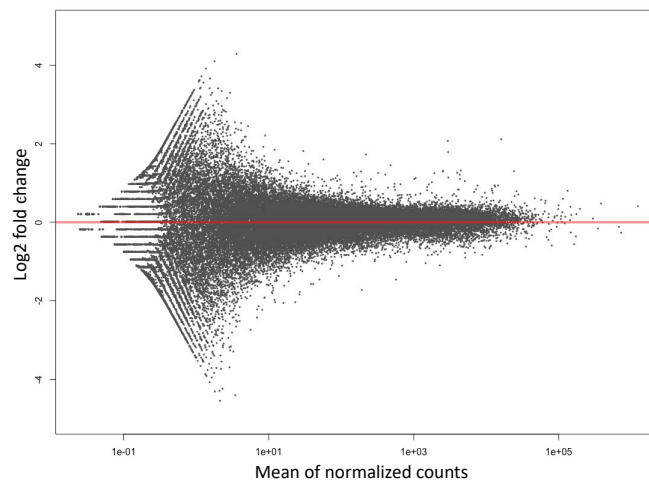


Figure 12.12: **MA-plot of the Null 4 hours RNA-sequencing data comparing differentially expressed genes between the low responders and responders.** Each dot represents one gene and red dots represents significantly differentially expressed genes (FDR < 0.01).

12.2.3.2 LPS 4 hours stimulation condition

To visualize sample-to-sample distances in the 4h LPS stimulation condition, we performed a PCA and colored each sample regarding the IL-12p70 response (Figure. 12.13A) and sex (Figure. 12.13B). From the PCA plots, we observed that 40% of the variability was represented by the first two principal components. Similar to the Null condition analysis, two clusters were present on the PCA plots and they were for the most part explained by sex rather than the IL-12p70 response.

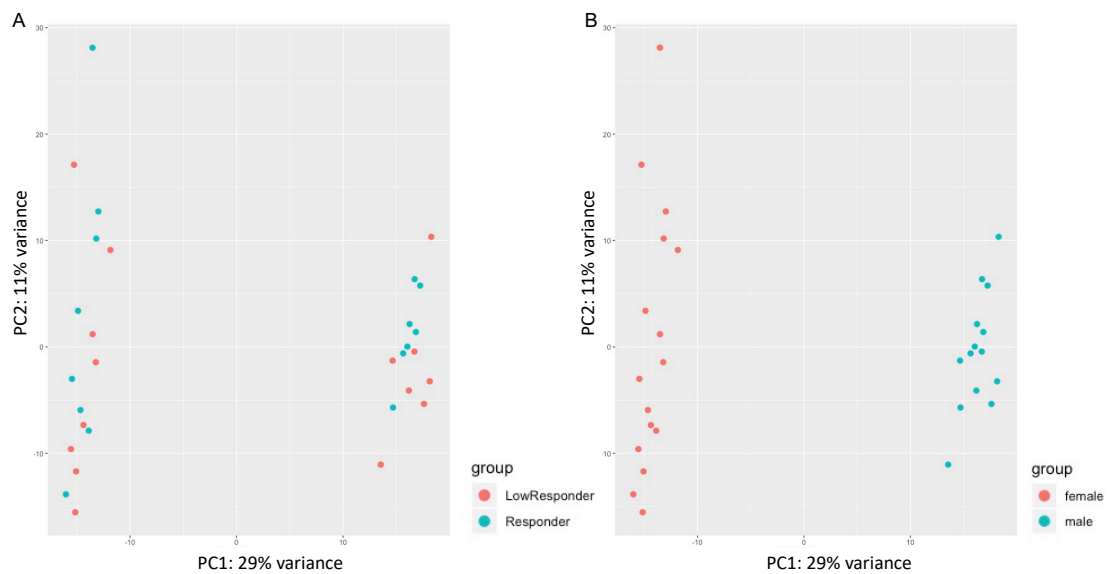


Figure 12.13: **LPS 4 hours stimulation condition principal component analysis (PCA) color-coded by IL-12p70 response (A) and sex (B).** Plots represent the first two components of the PCA including percentage of variance link to each axis.

Next, we performed DGE analysis between the two low responder and responder groups and found no significant differences for any of the genes ($FDR < 0.01$) (Figure. 12.14). Of note, the *IFNB1* gene was not significantly different between the two groups (data not shown) as observed at the protein level measured in the 4h LPS supernatants. Therefore, we did not obtain any information explaining the observed IL-12p70 phenotype from this dataset as expected.

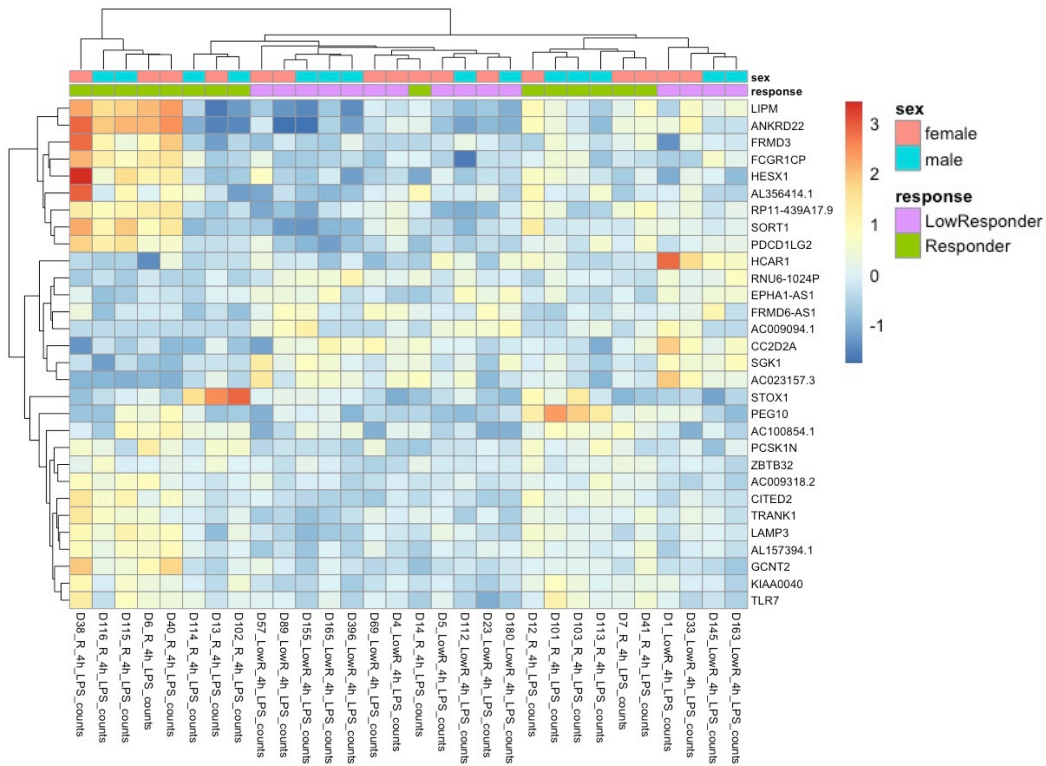


Figure 12.14: **Heatmap of the 30 most differentially expressed genes between low responders and responders in the LPS 4 hours stimulation condition.** Rows and columns were clustered using hierarchical clustering using variance-stabilized transformed values.

12.2.3.3 LPS 22 hours stimulation condition

Finally, we performed PCA and DGE analysis between the low responders and the responders using the 22h LPS stimulation condition RNA-sequencing data. One more time, samples clustered according to sex rather than the response condition (data not shown). The DGE analysis revealed that 1287 genes were significantly differentially expressed between the two groups ($FDR < 0.01$) with $IFN\gamma$ being the most differentially expressed gene ($FDR = 6.8e-05$) (Figure. 12.15). This result was consistent with the Nanostring DGE analysis and represents downstream effects of differential $IL-12p70$ production.

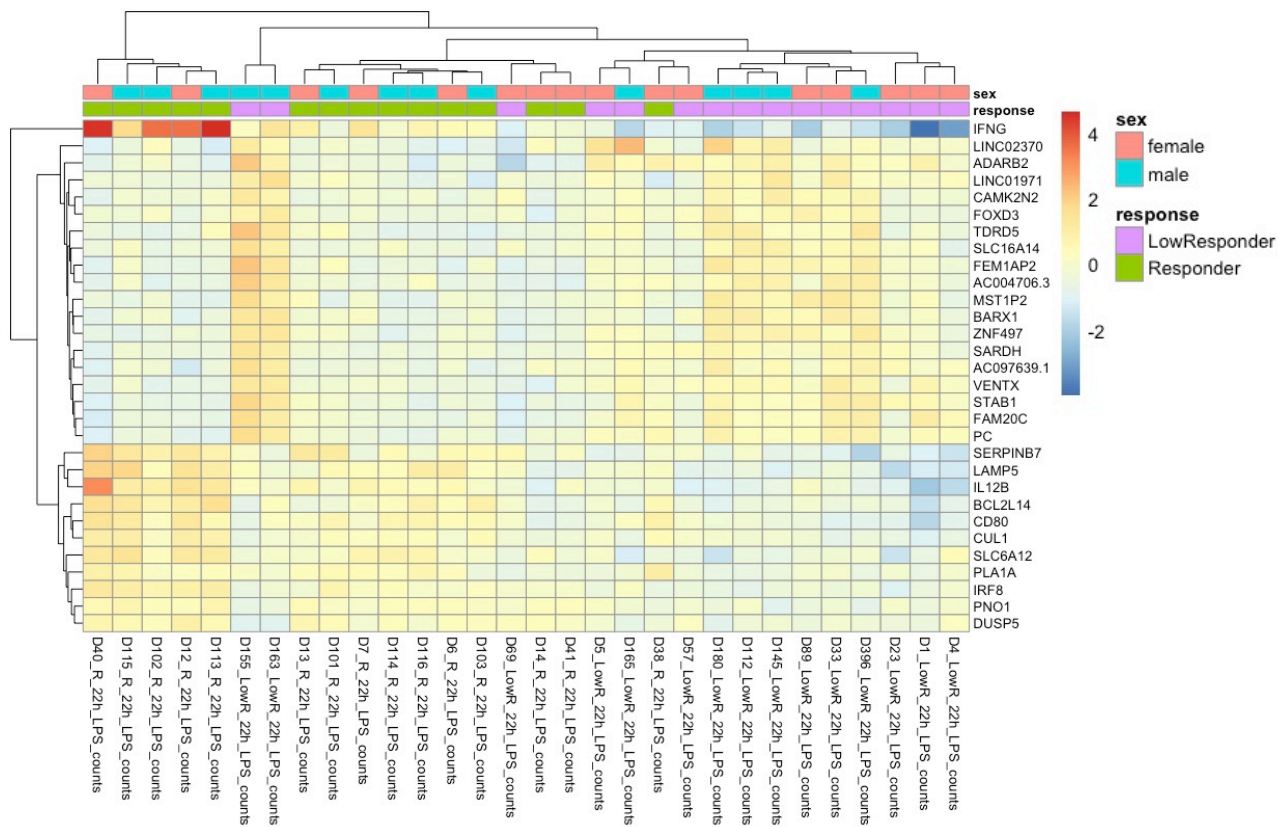


Figure 12.15: **Heatmap of the 30 most differentially expressed genes between low responders and responders in the LPS 22 hours stimulation condition.** Rows and columns were clustered using hierarchical clustering using variance-stabilized transformed values.

12.2.4 Conclusions and new hypotheses

No significant differentially expressed genes were detected between the low responders in the 4 hour Null and LPS conditions. Similar to the Nanostring data analysis, $IFN\gamma$ was significantly differentially expressed following LPS 22 hours stimulation. These results did not allow us to explain variability in $IFN\beta$ and $IL-12p70$ responses. From this analysis we can make two hypotheses. The first hypothesis is that the stimulation timepoint is not appropriate and more precisely that a 4 hour LPS stimulation is too long to observe gene expression differences upstream of $IFN\beta$ production between the low responders or responders. The second explanation is that the small number of donors sequenced could explain the lack of result obtained and we conclude that sequencing 15 donors in each group was not enough to observe differences. This last hypothesis is supported by

the following analysis. We subsampled the reads of 10 donors equally distributed regarding the IL-12p70 response to 120, 100, 80, 60 and 40 million reads. We performed DGE analysis and compared the significant number of differentially expressed genes between subsampled conditions. To analysis the impact of the number of donors sequenced, we also reported the significant number of differentially expressed genes using all 30 individuals (Figure. 12.16). First, we observed that 40 to 80M reads already capture most of the differentially induced genes between Null and LPS as compared to 100 to 120M reads. The 120 million read condition allowed to detect only 485 more genes differentially expressed than the 40 million read condition. We also noticed that the number of differentially expressed genes using all available reads (> 120M) of the 30 donors allowed to detect twice more genes differentially expressed than the analysis performed using 10 donors. This analysis suggests that the number of subjects highly impacts the number of detected genes and that increasing the number of donors sequenced might lead to identification of differentially expressed genes between the low responders and responders in the 4 hours LPS condition.

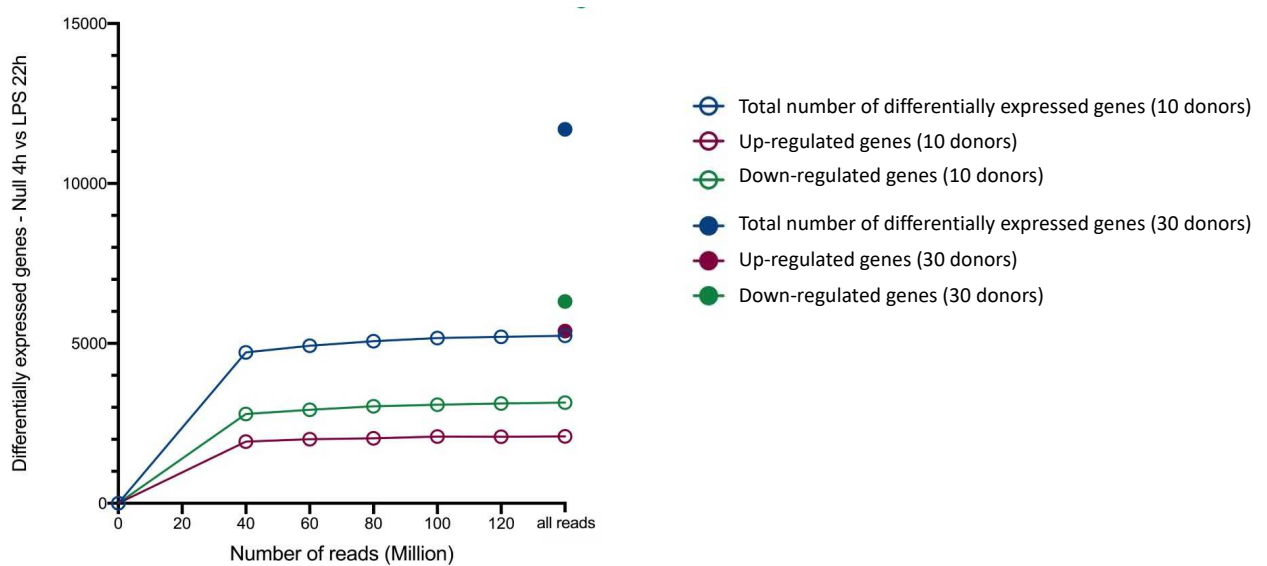


Figure 12.16: **Differential gene expression analysis based on different number of reads and number of donors.** Sequencing data were randomly subsampled for 10 donors (5 low responders and 5 responders) and differential gene expression analysis was performed between Null 4h and LPS 22h conditions. Total number of differentially expressed genes, up-regulated genes and down-regulated genes were reported according to the number of reads used for the analysis (FDR < 0.01). Significant number of differentially expressed genes were also reported using all reads available of the 30 sequenced donors.

Part IV

Discussion

In this study we aimed at dissecting immune response variability and more specifically explain naturally occurring variability of IL-12p70 cytokine in the context of bacterial infection among healthy European individuals. These 1,000 healthy donors are part of the *Milieu Interieur* project which aims to identify genetic and environment factors that contribute to the observed diversity of the immune responses. The main findings presented in this manuscript include:

- i) The characterization of a consistently low IL-12p70 response upon LPS whole blood stimulation in a quarter of the healthy population using an ultrasensitive immunoassay.
- ii) The finding that IL-12p70 secretion by monocytes and dendritic cells is dependent on monocyte IFN β production both in health and disease settings.
- iii) The identification of a novel genetic associations with both IL-12p70 and IFN β protein secretion specific to LPS stimulation.

Based on these findings we were able to test our principle hypothesis, namely that dissecting this natural variance in a healthy MI reference population will provide new insights into disease pathogenesis. To do that, we examined available patient cohorts from TB and HCV studies, which confirmed the importance of IFN β production for IL-12p70 secretion in both diseases. Furthermore, this perturbed response was observed at significantly higher frequencies in patients chronically infected with TB or HCV.

13. Strengths and limitations of our approach

13.1 Whole-blood TruCulture assay

The determination of cytokine secretion in health and disease conditions was performed using whole blood stimulated in TruCulture tubes. TruCulture tubes for healthy individuals and chronically infected TB or HCV patients were generated in three different centers. Comparison of induced immune responses across several centers was previously assessed using this whole-blood syringe assay system [17]. This study revealed that TruCulture tube is a pertinent immunomonitoring tool as high reproducibility across centers and reduced background cytokine secretion were observed. Thus, human whole blood stimulation in TruCulture tubes minimize technical variations between centers, is a good model to mimic *in vivo* infections and allows robust analysis of induced cytokine secretion. Different approaches can be used to characterize immune responses including animal models, single-cells, PBMCs or whole blood. Although whole blood stimulation does not necessarily provide information at the single cell level, the advantages of using whole blood are multiple. Blood collection is non-invasive and it contains all immune cells that circulate through all the body tissues. Unlike PBMCs, whole blood includes granulocytes and erythrocytes and its serum also contains all soluble factors that impact cell activation. As a consequence, whole blood stimulation is more biologically relevant reflecting *in vivo* conditions as it captures gene expression and protein secretion resulting from interactions of all circulating immune cells that occur during infection. The manipulation of whole blood also leads to more reproducible data as it minimizes sample manipulation required for single-cell, PBMCs isolation or cell culture. Lastly, animal studies might not always reflect human reactions to exposures [242]. Although whole blood stimulation is a good model, we acknowledge that using whole blood has some limitations and does not reflect the entire picture of a human immune profile. Performing research with other human tissues is also necessary to understand healthy human immune responses.

13.2 Cutoff used to define the responder and low responder groups

Wurfel et al., used seven different cytokines to define LPS low responders and responders identifying only 3 donors in each group. The classification was made based on seven cytokine concentrations all below the 40th percentile (LPS low responders) or above the 60th percentile (LPS responders) [243]. Here, we used the limit of detection of the Luminex assay (18.5 pg/ml) to define the two groups in the healthy MI cohort based on one single cytokine secretion which is IL-12p70. Using this cutoff, every donor of the MI cohort was assigned as a responder or a low responder and this classification was used as a qualitative variable for all subsequent analysis. This cutoff was also relevant to detect significant differences in 16 proteins out of 25 measured between the two groups.

13.3 Luminex *versus* Simoa immunoassay

Following from these initial observations made with Luminex, we measured IL-12p70 in all MI individuals using the ultrasensitive Simoa technology to define whether the donors for whom we did not detect any IL-12p70 were low responders or non-responders. Using the same antibody pair as used for the Luminex assay, we were able to detect induced IL-12p70 in LPS samples compared with the null condition, allowing us to define the donors as low responders. So far, the quantification of protein secretion was made by conventional immunoassay methods such as ELISA or Luminex. These techniques do not always provide sufficient sensitivity to fully detect cytokine production especially for baseline measurements. Indeed, cytokines are often present in low or sub pg/ml concentrations in human blood and are challenging to detect using these traditional assays [244]. The development of digital ELISA addresses this limitation [245]. This is clearly evidenced by our homebrew IL-12p70 Simoa assay being 1000-fold more sensitive than the Luminex IL-12p70 assay and allowed the characterization of IL-12p70 in all 1,000 MI donors in LPS and Null control tube supernatants. The comparative evaluation of IL-12p70 secretion between Luminex and Simoa showed a highly positive and significant correlation between the two techniques ($R=0.86$, $P < 0.0001$). The measurement of IL-12p70 in the same 1,000 samples by two different technologies and machine operators permitted to validate the “veracity” of the observed phenotype. Although IL-12p70 secretion was close the LOD of the Simoa assay in the null condition, the enhanced

sensitivity of the IL-12p70 Simoa assay allowed the detection of IL-12p70 in 672 donors in the null condition compared to only 2 donors detected using Luminex. Statistical analysis did not show any significant difference between the low responders and the responders in the null condition. Thus, we can hypothesize that baseline IL-12p70 levels do not differ between the two groups and that the observed phenotype is only present upon LPS stimulation. This is also supported by the gene expression results. In the null condition, the expression of both IL-12p70 subunit genes was not significantly different between the two groups.

13.4 Limitations of transcriptomic analysis using Nanostring

Transcriptomic analysis using Nanostring showed that the low responders significantly expressed less *IL12A*, *IL12B* and *IL23A* genes which encode for the p35, p40 and p19 subunits respectively. The Nanostring platform is based on direct digital quantification of the mRNA. The main benefit of Nanostring is to avoid bias as it does not require enzymatic amplification and library construction. In addition, it does not require high RNA quality (RIN > 3) compared with next generation sequencing (NGS) (RIN > 7). It also shows high reproducibility and robustness between data measured at different timepoints and using different facilities [246, 247]. However, the Nanostring technology presents some limitations. First, several immune pathways remain unexplored due to the restricted number of genes that can be measured. This introduces bias when performing gene set enrichment analysis and pathway analysis. While NGS allows identification of novel transcripts, the Nanostring platform does not provide information on splice variants and non-coding RNA induced after whole blood stimulation. The *IL12B* and *IL23A* genes induce only one transcript coding for their protein however, the *IL12A* gene can produce three different transcript variants coding for three isoforms of the p35 subunit (Ensembl and NCBI websites). Using Nanostring, we were not able to distinguish the different *IL12A* transcripts and define whether one of the phenotypes or one of the genotypes express more specifically one of the transcripts. This limitation can be overcome with high throughput sequencing which can be used as a complementary technique to Nanostring to validate our findings.

14. Other studies assessing cytokine secretion variability in humans

Several other studies investigated inter-individual cytokine secretion variability in healthy donors. As mentioned in the introduction, some large-scale studies focused on basal circulating cytokine levels in the blood whereas other studies assessed cytokine secretion variability following whole-blood stimulation. Enroth et al., measured 92 proteins including IL-12p70 in whole blood of 1,005 Swedish healthy individuals. As compared to the MI cohort, this cohort was not stratified by age and sex and the minimal age eligibility criteria was 15 years and did not include any maximal age. Two significant associations were found with IL-12p70 plasma levels. First, a local cis-QTL association was found. The rs10045431 SNP, located in the intergenic region of *IL12B* gene, was significantly associated with IL-12p70 secretion. Second, smoking affected IL-12p70 secretion. Smokers had less circulating levels of IL-12p70 than non-smokers in this cohort [6]. In the MI cohort, smoking was not found to be associated with IL-12p70 secretion following Null 22h stimulation (result not shown). This could be explained by the small number of donors for whom IL-12p70 was detected (n=672) or, most likely that the protein levels measured in the Null 22h TruCulture supernatants cannot be compared to baseline circulating levels from fresh whole blood. The MI cohort does not include children and teenagers and does not allow the study of cytokine variability below 20 or above 69 years of age. One recent study assessed cytokine secretion in 271 children from 1 month to 13 years old both in unstimulated and stimulated whole blood with phytohaemagglutinin (TLR₄ agonist) for 24 hours [248]. IFN γ was found to be significantly increased with age in this cohort. Using our model, we determined that age explained 0.5% of the IL-12p70 variability with a significant decrease of IL-12p70 secretion with age following LPS stimulation. As IL-12p70 secretion reflects IFN γ production, we can hypothesize that IL-12p70 and IFN γ production following TLR₄ activation reaches adult levels in the teenage years and slowly decline after 30 to 40 years old. This age-dependence of TLR induced Th₁ responses was also summarized and illustrated by Kollmann et al [249]. The “Human Functional Genomics Project” (HFGP) large-scale study (n= 489 healthy subjects) evaluated host and environmental factors that impact human cytokine produc-

tion variability. This study assessed variability in IFN γ secretion upon whole blood LPS stimulation but did not assess IL-12p70 responses and its related members [13]. The MI cohort includes 200 individuals per decade split equally by sex (1:1 ratio). This balanced experimental design provides a large statistical power as well as homoscedasticity. Although the HFGP study included a large number of healthy volunteers, the cohort was not well stratified by age and sex. A smaller study (n=102 healthy donors) previously defined LPS low responders and LPS responders based on cytokine responses following whole blood LPS stimulation [243]. Again, this study did not investigate IL-12p70 cytokine secretion. In addition, none of these studies used standardized immunomonitoring tools to investigate cytokine secretion variability in healthy individuals.

15. Potential Biological and Clinical significance of our findings

15.1 Extrapolation of the observed phenotype to gram negative bacterial infection

We also observed that the low responders secrete significantly less IL-12p70 than the responders following *E. coli* stimulation. LPS which was used to define the phenotype, is a compound of the outer membrane of the gram-negative bacteria *E. coli*. Of important note, *E. coli* infection is more complex than just LPS stimulation as it includes a peptidoglycan layer, membrane proteins and flagellin which triggers inflammation through its interaction with TLR₅ [250]. In addition, the use of *E. coli* reproduces the clinical situation of infection more closely than LPS stimulation. As an example, LPS and *E. coli* show differences in the activation of microglial cells in the brain with *E. coli* mimicking better the clinical situation [251]. Here, we found a positive and significant correlation for IL-12p70 production between LPS and *E. coli* stimuli ($R = 0.78$, $P < 0.0001$). As a consequence, even if LPS stimulus is more simplistic than *E. coli* stimulation, we can consider that our LPS model simulates the *in vivo* situation and reflects the Th1 response induced with an *E. coli* infection in human whole blood.

15.2 Endotoxin tolerance

To assess if the phenotype was stable over time, we measured IL-12p70 in null and LPS supernatants of 500 donors of the MI cohort who participated to visit 2 that occurred 2 to 6 weeks after visit 1. We found that the phenotype was persistent as the low responders secreted significantly less IL-12p70 than the responders upon LPS stimulation and IL-12p70 levels measured in visit 2 samples, which strongly correlated with the visit 1 IL-12p70 concentrations. One potential explanation for the observed phenotype could be that the immune cells of 28% of the healthy donors have been exposed to LPS previously to blood collection leading to endotoxin tolerance. Upon exposure to endo-

toxin, myeloid cells become refractory to subsequent LPS challenge. This phenomenon is named endotoxin tolerance and is associated with immunosuppression. On one hand, this reduced responsiveness to LPS protects against a lethal challenge of LPS and endotoxin shock. On the other hand, endotoxin tolerance is associated with immune dysfunction mediating sepsis pathogenesis [252]. Sepsis induces an initial inflammatory response which is then followed by an anti-inflammatory response acting as negative feedback. Endotoxin tolerance was investigated in humans and its duration was estimated at between two to five weeks [253, 254]. As visit 2 samples were collected two to six weeks after visit 1, we cannot completely exclude the hypothesis of LPS tolerance occurring in 28% of the cohort. Indeed, if the low responder donors were exposed to LPS prior to blood collection for the MI project, whole blood LPS stimulation both in visit 1 and visit 2 could have led to endotoxin tolerance and a decrease of IL-12p70 secretion. Several studies have demonstrated that endotoxin tolerance leads to a decreased production of pro-inflammatory cytokines including $\text{TNF}\alpha$, IL-6, IL-1 α , IL-1 β , CXCL10 and IL-12p70 and an increase secretion of anti-inflammatory cytokines such as IL-10, TGF β and IL-1RA in LPS-tolerized monocytes as compared with monocytes from healthy donors [255]. A decreased IL-12p70 secretion was associated with impaired Th1 cell proliferation and a shift toward a Th2 immune response [256]. In the MI cohort, $\text{TNF}\alpha$, IL-6, IL-1 α , IL-1 β and IL-12p70 cytokines were significantly less secreted in the low responder group following LPS stimulation supporting the LPS tolerance hypothesis. However, the anti-inflammatory IL-1RA cytokine was significantly less secreted in the low responders. In addition, the IL-10 cytokine was not significantly different in either group ($Q = 0.05$). Although TGF β was not investigated in the project, the anti-inflammatory cytokines were not highly produced in the low responder group and this is not in accordance with results reported in LPS tolerance studies. Future human studies, investigating cytokine variation upon bacterial infection, should ensure that the second blood collection occurs at least two months after the first sampling to study cytokine profiles overtime and exclude a potential role for endotoxin tolerance.

15.3 IFN β and IL-12p70 secretion in HCV and TB

We sought to apply our findings to relevant clinical cohorts, namely stimulated whole blood samples from both HCV and TB patient cohorts. Both of these diseases have previously been shown to have ISG signatures in the blood that are negatively correlated

with clinical outcome. In HCV infected patients, LPS significantly induced both IL-12p70 and IFN β compared to the null condition. However, no significant difference between the SVR- and the SVR+ patients were observed for these two cytokines neither in the null nor the LPS stimulation condition. Although IFN α and ISG transcriptional activity were not measured, this result suggests that IFN β -induced signaling and potentially ISG induction by IFN β were not different between the two groups in whole-blood. Type I IFN signature has been investigated both in liver biopsies and PBMCs pre and post treatment with IFN α in HCV infected patients. Patients who poorly respond to the therapy showed preactivation of their type I IFNs system before the treatment which was specifically observed in the liver biopsies but not in PBMCs [257]. Thus, levels of IFN β and IL-12p70 in whole-blood following null and LPS stimulations are not predictors of the response to the IFN α therapy. However, we observed that following LPS stimulation, IFN β and IL-12p70 were positively and significantly correlated specifically in the SVR+ patients but not in the SVR- patients. We can hypothesize that the induction of IL-12p70 by IFN β through TLR₄ activation is altered in SVR- patients. We did not observe any significant difference in the null condition between the 1,000 MI donors and the 100 HCV infected patients. This is not in accordance with the literature describing that HCV infected individuals have higher levels of IL-12p70 in the serum compared to healthy donors [229, 228]. Nevertheless, IL-12p70 was significantly more secreted in the MI donors compared to the HCV infected individuals following LPS stimulation ($P < 0.0001$) suggesting that healthy donors have a greater ability to induce a Th1 response upon bacterial infection than HCV infected subjects. It would have been interesting to measure IFN α to see how it correlates with IL-12p70 as compared to IFN β in HCV patients, as well as other interleukins. Indeed, IL-10 was shown to be associated to a non-response to the IFN α treatment or IL-18 which, like IL-12p70, was also associated with a sustained virological response [228]. In addition, IL-6 and IL-8 were also shown to be significantly higher in the serum of infected patients compared to healthy controls [230]. In TB infected individuals, we observed a significant difference in IFN β and IL-12p70 secretion between active TB and latent TB subjects in the Null condition. Both cytokines were more secreted in the latent TB group compared to the active TB group. These observed differences might be explained by technical challenges as we did not have access to the same volume of samples between latent and active individuals for cytokine measurements in the null condition. Therefore, they were tested at different dilutions which may affect the final concentration especially when close to

the LOD. These results are also not consistent with the literature. Wang et al., measured 27 cytokines including IL-12p70 in 28 active TB patients and 34 latent TB individuals and showed a significant increase of IL-12p70 in the active TB group compared to the latent group and TB uninfected controls [258]. Following LPS stimulation, both latent and active TB groups showed a significant and positive correlation between IFN β and IL-12p70 secretion. The correlation of IL-12p70 with IFN β was higher than the correlation between IL-12p70 and IFN α in the two groups confirming the IFN β /IL-12p70 relationship observed in the MI healthy donors. As these results showed that IFN β and IL-12p70 secretion correlate in the two TB groups, we can hypothesize that the TLR4 pathway leading to both IFN β and IL-12p70 secretion is not altered in any of the groups and that these two cytokines might not be potential biomarkers for discriminating between active TB patients and latent TB individuals.

16. Systems biology to integrate multiple diverse data sets

Complex networks allow the healthy immune system to maintain tissues homeostasis and to eliminate pathogens while avoiding tissues destruction. Systems immunology includes methods to measure and understand human immunity and obtain causal and predictive explanations of the observed immune responses in the context of health and disease. Systems immunology aims at discovering unknown relationships among variables to understand the immune system as a whole. These variables include environmental factors, clinical descriptors, genetic variants, immunophenotyping data, epigenetic states and others. The objective is to identify the most critical factors and interactions that drives immunity. This is made possible with the integration of clinical research, the development of innovative high throughput technologies, immunological expertise and advances in bioinformatics to analyze large data sets. These inter-disciplinary collaborations allow considerable progress in human translational medical research and aims to develop new tools in the clinic to both monitor and predict immune responses in health and diseases [259, 260].

16.1 Genetic influence on cytokine responses

Protein or cytokine quantitative trait loci analysis (pQTL and cQTL respectively) analysis can greatly help us understanding how genetic factors control human inter-individual variation in cytokine secretion. The role of genetic variation on inter-individual variability in cytokine responses in healthy subjects was previously investigated in several studies. Genetics can explain variability in baseline plasma cytokine abundance in healthy individuals. However, the inter-individual variability in plasma concentration of cytokines at baseline is for the most part driven by non-heritable factors with age and body weight affecting a large number of proteins [7, 6]. In contrast, the HFGP project has shown that genetic variation plays a major role in inter-individual variability of cytokine secretion in response to pathogen stimulation. After whole blood or PBMC stimulation with microbial stimuli, they demonstrated that genetic variants impact the production of six dif-

ferent cytokines [14, 261]. So far, none of these comprehensive genome-wide association studies has measured and investigated the impact of host genetics on IL-12p70 and type I IFN responses at the proteomic level. Using the genotyping data of the 1,000 MI donors, we performed a pQTL analysis to identify potential SNPs that could impact both IFN β and IL-12p70 protein secretion upon LPS stimulation. We detected 5 novel cis-acting SNPs associated with IL-12p70 secretion and 4 novel cis-acting SNPs significantly associated with IFN β production. These two groups of SNPs are in strong linkage disequilibrium (Ensembl website). First, we found a very significant association between the variants rs17753641, rs143060887, rs17810546, rs76830965, rs17809756 and IL-12p70 secretion ($Q < 4.32e-6$). These SNPs did not impact IL-12p40 or IL-23 cytokine secretion. Interestingly, they also did not affect *IL12A*, *IL12B* and *IL23A* gene expression as measured by Nanostring. These variants are all located upstream of the *IL12A* gene and inside the antisense *IL12A-AS1* gene. *IL12A-AS1* is an antisense long non-coding (lncRNA) that can bind to the *IL12A* mRNA and inhibits its translation into functional protein. lncRNA can control nearly every level of gene regulation through DNA-RNA, RNA-RNA or protein-RNA interactions [262]. Four of these SNPs are intron variants and non-coding transcript variants that overlap with 8 different *IL12A-AS1* transcripts. They are also located in a promoter flanking region and within the H3K27Ac histone mark (acetylation of lysine 27 of the H3 histone protein) (UCSC website). This mark is thought to enhance transcription possibly by blocking the repressive histone mark H3K27Me3. The last SNP, rs17809756, overlaps only 3 transcripts of *IL12A-AS1* and is defined both as an intron variant and as a non-coding transcript exon variant. It remains to be established how the SNPs impacts IL-12p70 secretion mechanistically. As *IL12A* and *IL12B* subunit gene expression are not impacted by the SNPs, the most probable hypothesis is that these variants might affect the p35 subunit translation. As the p35 subunit is not secreted on its own in contrast to p40, p35 production cannot currently be assessed in human cell culture supernatant. However, p35 is also a constituent of the IL-35 cytokine. The development of an IL-35 Simoa assay and measurement of IL-35 in the supernatant of the 1,000 MI donors would allow us to assess whether the SNPs are also associated with IL-35 secretion. Second, we observed that rs10757182, rs10811473, rs13291085 and rs7020908 cis-acting SNPs were significantly associated with IFN β secretion. These four variants are intergenic SNPs located between the *IFNB1* and *IFNWP4* genes on chromosome 9. As for the IL-12p70 associated SNPs, none of the IFN β associated SNPs impacted *IFNB1* gene expression measured by Nanostring upon LPS stimulation, which may be

due to kinetic differences between protein and RNA expression. Experimental mechanistic studies should be performed to understand how these 4 variants specifically impact IFN β secretion. The results of our model showed that the identified factors impacting IL-12p70 secretion explain 32.8% of the variability; with IFN β secretion alone explaining 21.6% of the observed variability. This highlights the need to study upstream immune pathways when dissecting variability in cytokine responses. Using our model, we could also determine that the five IL-12p70 associated SNPs explain 4.2% of IL-12p70 secretion variability following LPS stimulation. Applied to IFN β secretion variability, the model revealed that, out of 11.8% of the total variability explained, the four SNPs only explain 2.3% of the variability. These values are lower than the median variability (10%) that we explained by genetics for induced gene expression in the MI cohort [21]. This approach showed that host genetics did not predominantly influence cytokine secretion variability following LPS stimulation. This is not in accordance with Schirmer et al, who discussed the impact of genetic and non-genetic factors on cytokine secretion and concluded from reviewing several studies that genetic factors explain a major part of cytokine secretion variability upon pathogen stimulation whereas non-heritable factors mainly drive cytokine variations in baseline condition [15]. We have to keep in mind that we might have missed some genetic influence on the cytokine secretion variability as the performed genotyping experiments did not capture insertions/deletions. Future analysis using whole-genome sequencing data will help to complete conclusions regarding the influence of host genetics on IL-12p70 and IFN β cytokine variability upon LPS stimulation.

16.2 Other proteins significantly differentially secreted between the two groups

Upon LPS stimulation, the transcriptomic analysis of the p40 and p19 subunits showed significant differences between the low responders and the responders suggesting that at the protein level, IL-12p40 and IL-23 cytokine secretion are also impacted. An expanded differential analysis of protein secretion between the low responders and the responders revealed that, out of 25 tested proteins, 17 proteins showed significant differences between the two groups (including IL-12p70, IL-12p40 and IL-23). Interestingly, we observed some associations between interferons and the majority of these cytokines in the literature. ENA-78 (also named CXCL5) is the only protein upregulated in the low re-

spander group as compared with the responder group. Both IFN α and IFN γ down-regulate ENA-78 in LPS stimulated human monocytes [263]. The secretion of ENA-78 may be upregulated in the low responders as they secrete less IFN β leading to less IL-12p70 resulting in less IFN γ secretion than the responders. IL-1 β , IL-1RA and TNF α cytokines, which are more produced in the responders, can be enhanced by IFN β in human monocytes activated by LPS [264]. However, the same study did not observe any impact of IFN β on IL-1 α and IL-6 cytokines for which we observed an upregulation of their secretion in the responder group. This might be due to different models used: purified monocytes *versus* whole blood. The induction of IL-6 might not be directly linked with IFN β but with IFN γ as Fujisawa et al., showed that IFN γ induces IL-6 production in a dose dependent manner in human keratinocytes [265]. Regarding the IL-15 and the BDNF cytokines, mouse DCs produce IL-15 following stimulation with type I IFNs [266] and IFN β has been shown to upregulate BDNF levels in PBMCs of multiple sclerosis patients [267]. These papers are also consistent with our results showing a higher secretion of IL-15 and BDNF in the responder group. Finally, a relationship between type I IFNs and the SCF complex was also previously described in the literature. The regulation of cellular responses to type I IFNs is mediated through the degradation of the IFNAR $_1$ receptor subunit which is dependent on SCF complex activity [268]. The VEGF protein seems to have the same effect as SCF. It promotes the degradation of IFNR $_1$ to attenuate type I IFN signaling [269]. However, the literature does not support our results highlighting higher secretion of the MMP-9 protein in the responder group. In human astrocytes and astrogloma cell lines, both IFN β and IFN γ inhibit MMP-9 gene expression [270]. In conclusion, this analysis means that the level of IFN β secretion might not only impact IL-12p70 but several other cytokines and this hypothesis is strongly supported by the literature.

16.3 Cellular analysis

To define which cell types produce IL-12p70 and IL-23 in whole blood following LPS stimulation we utilized intracellular flow cytometry as the most sensitive cell based approach. Regarding the IL-23 cytokine production following LPS stimulation, 41% of monocytes and 19% of cDC $_2$ secreted IL-23. In comparison, 7% of monocytes and 11.5% of cDC $_2$ specifically secreted IL-12p70. As human blood contains 10 times more monocytes than cDC $_2$, we can deduce that monocytes are the main producers of the IL-12p70

cytokine in whole blood upon LPS stimulation. In addition, similar percentages of monocytes and cDC2 (25%) secrete both IL-12p70 and IL-23 simultaneously. These observations mean that monocytes are the main producers of both IL-23 and IL-12p70 in whole blood and that the majority of cells that secrete IL-12p70 also produce IL-23 at the same time. The co-expression of both cytokines in the same cells might lead to competition between the p35 and p19 subunits for binding to p40. Indeed, even though several residues of the p40 chain are used specifically for each subunit interaction to form the heterodimers, the majority of p40 residues that are in contact with p19 are also in contact with the p35 subunit [271]. However, knowing that the IL-12p40 cytokine is produced in excess and that the p35 subunit is the limiting factor for IL-12p70 secretion [272], we can assume that p19 and p35 may not compete for binding to p40. It remains to be established by future studies if one of the subunits has a higher affinity for p40. Following from this observation, we extended our analysis to all the immunophenotyping data available for the 1,000 MI donors to investigate whether the low responders had less circulating monocytes and DCs than the responders at basal state. We did not observe any significant differences excluding the hypothesis that the phenotype is driven by differences in monocyte and DCs cell numbers. We also performed in parallel an unbiased comparison between low responders and responders of all MFI and cell number phenotypes. The results of the unbiased comparison revealed that the monocytes and cDC2 of the low responders had significantly less HLA-DR expression on their surface than the responders. The CD86 co-stimulatory marker was also less expressed at the surface of the CD14⁺ monocytes of the low responders. HLA-DR is an MHC class II cell surface receptor mainly present on APCs. APCs use HLA-DR to present peptide antigens to the T cell receptor to induce an appropriate adaptive immune response. CD86 is also constitutively expressed on the surface of APCs and provides co-stimulatory signals for T cell proliferation and generation of cytotoxic T cells through its interaction with CD28 on T cells [273]. HLA-DR and CD86 are both necessary for interactions between APCs and T lymphocytes to induce specific immune responses to a foreign antigen. Low HLA-DR levels are associated with immunosuppression and have been linked with immune failure. HLA-DR marker is an independent predictor of mortality in human septic shock [274]. The expression of CD86 on monocytes has also been reported to be decreased in critically ill patients with sepsis compared to control subjects leading to impaired antigen presentation and altered immune responses [275]. From the literature, we can hypothesize that either the low responders have been exposed to in-

flammatory mediators previous to blood collection leading to immunosuppression and a decreased HLA-DR and CD86 levels as previously discussed in regards endotoxin tolerance. Alternatively, this basal low level of HLA-DR and CD86 is not due to endotoxin tolerance and might increase susceptibility of the low responder individuals to complications in the context of septic shock due to a hypo-inflammatory response. It might also increase the incidence of infection and affect immune responses during pathogen infection. Indeed, low expression of HLA-DR and CD86 have been associated with impaired cellular functions of monocytes including a reduction of their pro-inflammatory properties and a decrease of their ability to induce T cell responses through reduced antigen presentation [276, 277]. In addition, both IFN β and IFN γ upregulate the expression of CD86 on human monocytes and DCs [278, 279, 280]. Also, the inactivation of CD86 on human monocytes significantly reduces IFN γ secretion by T cells [281]. The low responders already have low levels of CD86 and secrete less IFN β upon LPS stimulation. This feedback loop probably maintains low levels of CD86 co-stimulatory marker and does not enhance immune functions such as T cell activation comparable to the responders.

17. Importance of kinetic studies for determining the roles of type I and II IFN on the low cytokine response phenotype

Upon LPS stimulation, 332 differentially expressed genes were identified between the two donor groups ($Q < 0.01$). *IFNG* was the most differentially expressed gene with a higher expression in the responder group. This observation was consistent with the protein data analysis as IFN γ was significantly less secreted in the low responder group following 22 hours of LPS stimulation. It is well known that IL-12p70 induces IFN γ production [26, 238]. Nevertheless, it has also been reported that optimal expression of p35 and p40 subunits in purified human monocytes by LPS requires specific priming by IFN γ [231] and IFN γ by itself induces gene expression of both subunits [24]. Moreover, Kraaij et al., demonstrated using both real-time PCR and flow cytometry that upon stimulation with LPS, monocytes secrete IFN γ [239]. Thus, we investigated whether IFN γ could be secreted upstream of IL-12p70 by monocytes and DCs and be responsible for the observed phenotype. To test this hypothesis, we first checked that monocytes and DCs could secrete IFN γ using intracellular flow cytometry after whole blood LPS stimulation. Although we tested two brefeldin A timepoints (at the stimulation time and 8 hours post stimulation), we did not observe any IFN γ secretion and could not confirm the results of Kraaij et al. We also measured IFN γ protein after 4 hours of LPS stimulation in 50 low responders and 50 responders and did not observe any significant differences between the two groups suggesting that IFN γ was not responsible for the phenotype observed after 22 hours of stimulation. These results did not support our initial hypothesis and indicate that the differential gene expression analysis performed using Nanostring after 22 hours of LPS stimulation reflects downstream consequences of IL-12p70 secretion. As such they helped us to refine our hypothesis and lead us to conduct the kinetic studies of IL-12p70 and interferon responses both at the mRNA and protein levels. This approach allowed us to confirm that IFN γ secretion was downstream of IL-

IL-12p70. Although the number of donors was limited, 3 out of 5 donors showed IFN γ secretion downstream of IL-12p70 secretion and for one donor (#2) both IL-12p70 and IFN γ secretion began at a similar timepoint. The transcriptomic kinetic analysis showed that *IFNG* gene expression was also downstream of *IL12A* and *IL12B* gene expression. The peak for *IFNG* gene expression was observed at 16 hours post stimulation for all donors whereas the highest expression for *IL12A* and *IL12B* genes was observed at 4 hours post stimulation. All things considered, these results do not support the hypothesis that upon LPS stimulation, in the low responder phenotype, monocytes secrete less IFN γ than the responders inducing less IL-12p70 secretion in the low responders. IFN α secretion was also detected downstream of IL-12p70 secretion for 3 out of 5 donors (donors 1, 4 and 5) whereas the induction of IL-12p70 in all 5 donors began 30 minutes to 1 hour after IFN β secretion. The time series analysis showed that IFN β is highly significantly associated with IL-12p70 secretion in contrast to IFN α and IFN γ . This analysis was also performed using the transcriptomic data of interferons and IL-12p70 subunits. *IFNB1* and *IFNG* genes were significantly associated with *IL12A* and *IL12B* gene expression and *IFNA2* was significantly associated with the *IL12B* gene. This investigation did not allow us to find any clear association of one interferon with IL-12p70 subunit gene expression highlighting that mRNA expression does not always reflect protein secretion. The association of IFN β with the phenotype was confirmed by measuring IFN α and IFN β in null and LPS TruCulture supernatants after 4 hours of stimulation. Furthermore, blockade of IFNAR1 type I IFN receptor subunit but not IFNAR2 completely abolished IL-12p70 secretion. Here, with three independent experiments, we demonstrated that IFN β induced by LPS is necessary for IL-12p70 secretion and is responsible for the IL-12p70 low responder and responder phenotype. De Weerd et al., showed that IFN β can signal through IFNAR1 independently of IFNAR2 and induce a particular set of genes that do not require STAT1 activation. More than 100 genes are specifically induced by the complex formed by IFN β and IFNAR1 in the absence of IFNAR2 [122, 121]. Gauthier et al., stimulated human monocyte derived DCs (moDCs) with LPS in the presence of IFNAR2 blocking antibodies and observed a significant decrease but no inhibition of IL-12p70 production [53]. Our findings are in accordance with these two studies and highlight that specific IFN β signaling through IFNAR1 is essential for IL-12p70 production following LPS stimulation. Other published studies contradict the enhancing role of type I IFNs on IL-12p70 or IFN γ production concluding an inhibiting role of type I IFNs on IL-12 production by DCs and Th1 responses [282, 283, 280, 284]. This discrep-

ancy is likely due to the models used (moDCs were used in most of these studies) as well as the time of exposure. Indeed, IFN β mediates opposing effects on IL-12p70 and IFN γ production depending on the DC maturation stage. In the presence of immature DCs and during DC maturation, IFN β promotes IL-12p70 production and Th1 responses. However, exposure of mature DCs to IFN β inhibits Th1 responses and promotes the secretion of the anti-inflammatory IL-10 cytokine [91, 285]. In addition, some studies based their conclusion on the ability of type I IFNs to suppress IL-12p70 secretion based on the IL-12p40 subunit behavior [286, 287, 282] for which the production does not always follow IL-12p70 secretion as discussed in the subunit regulation part below. Proteomic measurements also revealed that IFN β and IFN α on their own do not induce IL-12p70, IL-12p40 and IL-23 secretion. However, both type I IFNs amplify IL-12p70 cytokine secretion when combined with LPS with IFN β inducing significantly more IL-12p70 than IFN α . In contrast, the addition of type I IFNs to LPS did not amplify neither IL-12p40 nor IL-23. The enhanced IL-12p70 production by addition of both type I IFNs to LPS was also observed by Gautier et al., and by Heystek et al., in a dose dependent manner in moDCs [53]. This protein analysis strongly suggests that type I IFNs induce specifically one of the IL-12p70 subunit. Following from this observation, we extended our research about IL-12p70 secretion regulation at the subunit level. Except for the p40 subunit, the study of the p35 and p19 subunits regulation cannot currently be performed at the proteomic level, but only at the gene expression level. Unfortunately, IFN β stimulation at 4 hours was not included in the original panel of stimuli of the MI study. We demonstrated that IFN β was induced following IFN α whole blood stimulation and *vice versa*. In addition, both IFN α and IFN β in combination with LPS enhance IL-12p70 secretion and none of these type I IFNs induce IL-12p70 secretion on its own. Thus, we believe that IFN α stimulation reflects IFN β stimulation and examined the expression of p35, p40 and p19 subunits after IFN α , LPS and LPS+IFN α 4 hours stimulation. IFN α stimulation induced *IL12A* but not *IL12B* and *IL23A* gene expression showing that type I IFNs induce specifically the p35 subunit. This is also in line with Gautier et al., who demonstrated a reduced expression of the p35 subunit gene expression in IFNAR $^{-/-}$ mice showing the role of type I IFN in the transcriptional activation of the p35 gene [53]. Interestingly, the co-stimulation of LPS with IFN α enhanced *IL12A* gene expression but reduced both *IL12B* and *IL23A* gene expression as compared with the LPS stimulation. This was also observed at the proteomic level where IL-12p40 and IL-23 secretion was decreased following LPS+IFN α stimulation in contrast to LPS stimulus. It seems that type

I IFNs inhibits p40 and p19 transcription. This observation is consistent with previous studies showing that the addition of type I IFNs to LPS stimulated human monocytes reduces IL-12p40 production [287]. Whether p40 and p19 subunits are directly or indirectly induced by LPS still remains to be determined. Taken together, these results allow to better dissect and understand IL-12p70 production under LPS whole blood stimulation in healthy donors.

17.1 Could the low responders be slow responders?

An alternative hypothesis is that the low responders are just slow responders and that they require more stimulation time to produce similar quantities of cytokines as the responders. Testing the hypothesis whether low responders are slow responders, would require to measure IL-12p70 and other differentially secreted cytokine in the MI cohort with a stimulation time longer than 22 hours. However, no stimulations were performed beyond 22 hours for the MI cohort so this cannot currently be performed to test the hypothesis. Our kinetic study included a timepoint of 30 hours of LPS stimulation. Although data are available for only 5 donors, we can clearly observe that, except for donor 1, all measured cytokines reached their plateau at 22 hours of stimulation and that 8 more hours of stimulation does not increase any cytokine concentration. This is also observed at the transcriptomic level. As previously mentioned, the peak for *IFNG* gene expression was observed at 16 hours post stimulation for all donors whereas the highest expression for *IL12A* and *IL12B* genes was observed at 4 hours post stimulation. Although we cannot completely exclude that the IL-12p70 low responders are slow responders, it is unlikely that a quarter of the healthy population take longer time to produce the IL-12p70 cytokine in their blood as the mRNA expression for both subunits occurs early after the LPS stimulation. This conclusion is also supported by the literature. Hayes et al., assessed p40 and p35 gene expression by Northern blot at different time points (from 0 hours to 8 hours) following monocyte stimulation with LPS. Although the timepoint did not exceed 8 hours, p35 and p40 mRNA accumulated very quickly. The p40 chain started to be expressed at 2 hours and continued to accumulate up to 8 hours whereas the p35 mRNA reached a plateau at 2 hours which was maintained over the time course [231]. This study also indicates an early induction of the expression of both IL-12p70 subunits. Regarding the HFGP project, Horst et al., stimulated whole blood for 48 hours before assessing all cytokine secretion by ELISA. Similar to the MI project, the IFN γ cytokine was

measured following whole blood LPS stimulation. The study presents IFN γ secretion according to oral contraceptive and gender using boxplots. From this data we can observe IFN γ secretion varies from 100 to 1000 pg/ml in average [13]. In comparison, IFN γ secretion data of the MI study measured by Luminex after 22 hours of stimulation varies from 100 to 4000 pg/ml on average. This timepoint comparison for IFN γ secretion suggests that 22 hours of stimulation is a reasonable timepoint to capture cytokine secretion induction and that a later stimulation timepoint would not lead to higher IFN γ production. We acknowledge a direct comparison using both datasets would be more precise to make any conclusions but it remains a good indicator that one additional day of stimulation will not necessarily leads to higher cytokine concentrations.

Part V

Conclusion and perspectives

18. Concluding remarks

The aim of this PhD project was to dissect the natural IL-12p70 occurring variance in the immune response at the level of population-based studies. This work has been performed in the context of the MI project that aims to assess the determinants underlying human immunological heterogeneity within the healthy population. We observed that a quarter of the healthy population consistently secreted low levels of IL-12p70 cytokine produced by monocytes and dendritic cells upon LPS stimulation. Although genetic factors and cellular stimulatory markers contributed to explain the IL-12p70 secretion variability, we found that 21.6% of the variance was determined by upstream monocyte IFN β secretion. From this work, we could extrapolate that specific IFN β signaling through IFNAR1 is key for triggering a Th1 immune response in bacterial infection. This dependent relationship between IFN β and IL-12p70 is of particular interest in the clinic. Indeed, we observed a perturbed IFN β /IL-12p70 association following whole-blood LPS stimulation of HCV infected patients who did not respond the IFN α -based therapy. In immunosuppression-associated pathologies, IFN β which induces the p35 subunit of IL-12p70 could be used in combination with a specific p40 subunit inducer to activate IL-12p70 secretion in patients showing deficient Th1 response. Novel genetic associations with both IL-12p70 and IFN β protein secretion specific to LPS stimulation were found in this study in which we also demonstrated that both cytokines were mainly produced by monocytes. It has been shown that monocyte-derived cQTLs are linked to susceptibility to infections whereas T cell-derived cQTLs are associated to autoimmune diseases [14]. Thus, we can hypothesize that the healthy MI low responder individuals might be more susceptible to infections. This hypothesis could be evaluated with the next stage of the *Milieu Interieur* project in which the majority of the MI donors will be resampled more than 10 years after the first project. This will allow to check the consistency of the IL-12p70 phenotype and to potentially assess the long-term impacts of this phenotype on their health by screening their immunological memory and risk of disease. Some determinants remain to be identified as all the variance was not explained according to our model. Our systems immunology approach should be enlarged using whole genome sequencing, RNA-sequencing and microbiota composition datasets. Whole genome sequencing might allow to identify new insertions deletions genetic associations with the

phenotype that were not investigated in the genotyping dataset. Using the 4 hour stimulation timepoint, RNA-sequencing should be extended to more donors. A power analysis performed to determine the sample size required to detect the IFN γ effect with a 95% degree of confidence highlighted that 105 donors in each group were required to observe a difference between the low responders and responders. We performed RNA-sequencing for only 15 donors per group giving us a 14,7% chance to detect difference between the two groups. In addition, investigating the impact of small non-coding miRNA on the phenotype might also give more insights on the observed variance as they regulate gene expression post-transcriptionally by binding to target mRNA that is then silenced or degraded. Finally, it would be interesting to test the impact of microbiome variation on the phenotype as it plays a key role in the education of the immune system and the establishment of immune tolerance against gut microorganisms. Overall, our work allows to classify healthy subjects in two groups regarding their Th1 response to TLR₄ activation and provide new finding about how IL-12p70 is produced and regulated upon bacterial infection both in health and disease settings.

Part VI

Methods

19. *Milieu Interieur* cohort – data analysis

To initially study variability in IL-12p70 cytokine responses, we took advantage of data previously established as part of the *Milieu Interieur* (MI) study. Relevant information regarding how these data was generated is described.

19.1 Donor recruitment, clinical and biological collections

The 1,000 healthy donors of the *Milieu Interieur* cohort were recruited by BioTrial (Rennes, France) from September 2012 to July 2013. This cohort is stratified by age and sex. 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 comprising biological and socio-demographic parameters [19]. The recruitment was restricted to individuals from metropolitan French origin for three generations to maximize statistical power for genetic analysis. Donors were defined as healthy according to medical history, clinical examination, laboratory results, and electrocardiography. Moreover, 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). Whole blood, nasal swabs and stool samples were collected for the 1,000 donors during visit 1 (v1). Half of the individuals, stratified by sex and age, were randomly selected to return for a second visit (v2) for repeat sampling to provide biological replicates for the validation of phenotypic studies. Visit 2 took place between 2 to 6 weeks after visit 1. Blood sampling included i) 20 mL of whole blood into heparin tube for flow cytometry study, PBMC isolation and plasma collection, ii) 5 mL of whole blood into EDTA tubes for DNA extraction and banking, iii) 60 mL of whole blood to study the induced immune response using TruCulture tubes (Figure. 19.1).

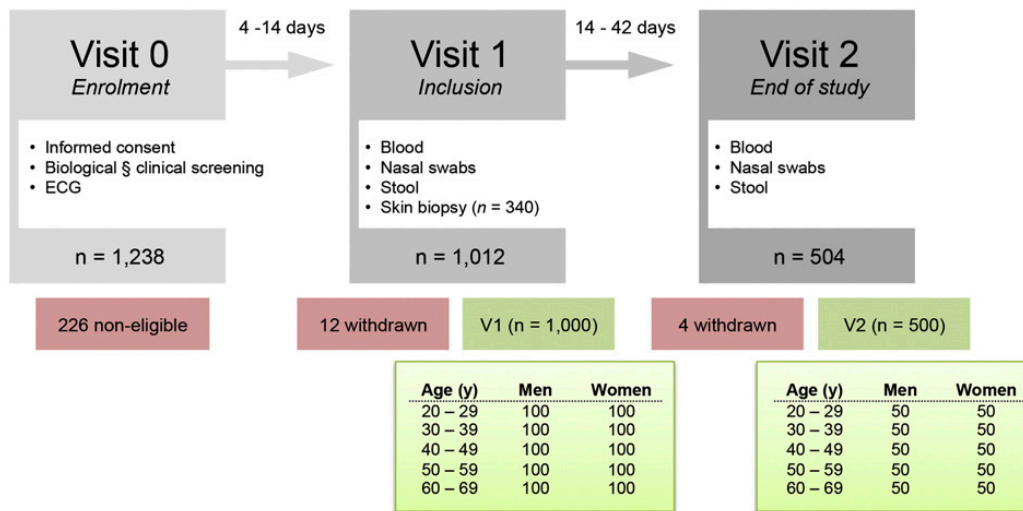


Figure 19.1: **Schematic representation of donor recruitment for the *Milieu Interieur* study.** To include 1000 healthy persons stratified according to sex (500 men, 500 women) and age (200 donors per decade of life, 20–69 years of age), we enrolled a total of 1238 individuals at visit 0 (Enrollment). Of those screened, 226 donors were considered non-eligible for reasons of consent withdrawal (n = 54), past medical history (n = 67), identification of an exclusion criteria during the onsite physical examination (n = 54), or during laboratory testing (n = 51). An additional 16 donors withdrew consent in the course of the study. During visit 1, whole blood, fecal samples and nasal swabs were collected. Punch biopsies of the skin were obtained from 340 of these donors. Half of the subjects were randomly selected (stratified by age and sex) to return for a visit 2, when repeat sampling of whole blood, fecal samples and nasal swabs was performed. Detailed medical histories and questionnaires were completed from all donors, recorded by medical personnel using an electronic case report form [19].

19.2 Whole blood Immunophenotyping

Whole blood was collected from the 1,000 healthy fasting subjects and delivered to Paris within 6 hours of blood draw and maintained at a temperature between 18°C and 25°C. Ten flow cytometry panels of eight-colors each were developed in order to enumerate and phenotype the major circulating leukocyte populations (T cells, Treg, Th cells, B cells, MAIT/NKT cells, NK cells, ILCs, PMNs (polymorphonuclear leukocytes), monocytes and DCs). Premix of antibodies were made manually every day and staining protocol was performed using the Freedom Evo 150 liquid handling system (Tecan). Samples were acquired using two MACSQuant analyzers. Panel antibodies and gating strategies were done as previously reported [288]. 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 [20].

19.3 Genome-wide DNA 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). Data were removed from the data set when:

- The SNP call rate was lower than 97%
- SNPs were unmapped on the public database dbSNP₁₃₈
- SNPs were duplicated
- SNPs had a low genotype clustering quality
- SNPs had a call rate <99%
- SNPs were monomorphic or on sex chromosomes
- SNPs were in Hardy-Weinberg disequilibrium

A final dataset of 5,265,361 SNPs was obtained.

19.4 Whole genome DNA methylation profiling

Extracted genomic DNA was treated with sodium bisulfite. Methylation levels of bisulfite-converted DNA were measured using 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. Raw IDAT files were first pre-processed with the minfi R package to perform quality controls. One sample showed high genotype discordance with the genome-wide SNP array data and was excluded and a total of 2,930 probes with > 1% missingness were also excluded. Using the irlba R package, Principal Component Analysis (PCA) of M values identified nine outlier samples which were also excluded. Batch effect was also corrected and outlier DNAm values were removed. Finally, we excluded (i) 83,380 non-specific probes that share > 90% sequence identity with several genomic regions, (ii) 118,575 probes that overlap a SNP with MAF > 1% in the *Milieu Interieur* cohort or in European populations from the 1,000 Genomes project, and (iii) 558 probes that were absent from the Illumina annotations version 'ilm10b4'. As a result, the final, quality-controlled data was composed of 969 donors profiled at 661,393 CpG sites. In order to identify methylation level differences between responders and low respon-

ders, 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 regress out any methylation level differences due to cell population differences between donors. The analysis was implemented using the R package “broom” vo.5.2. Multiple testing correction was then applied to select the significant genes.

19.5 Whole blood TruCulture stimulations

TruCulture tubes (Myriad RBM) containing 2ml of buffered media and stimulant were thawed at room temperature and 1ml of fresh blood was distributed into each tube previously labelled. Tubes were mixed by inverting them and incubated at 37°C for 4 or 22 hours 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 into 2D barcoded tubes (Matrix ScrewTop tubes, ThermoFisher) 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.

19.6 Protein quantification using Luminex

Supernatants from whole-blood TruCulture supernatants were analyzed by Myriad RBM using the Luminex xMAP technology. Samples were analyzed according to the Clinical Laboratory Improvement Amendments (CLIA) guidelines. TruCulture supernatants were added to wells of a plate containing a mixture of color-coded beads conjugated with protein-specific capture antibodies that bind to the analytes of interest. Biotinylated detection antibodies that are also specific to the proteins of interest were added to each well followed by PE-conjugated streptavidin that binds to the detection antibodies. Finally, beads were read with the LX200 instrument. The lower limit of quantification (LLOQ) was defined based on the standard curve for each analyte. It corresponds to the lowest concentration of a protein in a sample that can be reliably detected. The lower assay limit (LAL) is the lowest value displayed after application of the standard curve and use of curve-fitting algorithms. For data mining, individual values below the LAL were replaced with a value that is 50% of the lowest value measured in the data set. This calculation was used as the limit of detection (LoD) for this project.

20. *Milieu Interieur* cohort – data generation

To further study variability in cytokine responses we utilized samples from the *Milieu Interieur* study to generate new data sets. Relevant information regarding how this data was generated is described.

20.1 Gene expression assays

20.1.1 High-throughput standardized RNA extraction

A random selection of 96 tubes was performed before each round of extraction as samples were extracted in groups of 96. Cell pellets in Trizol LS of TruCulture tubes were thawed on ice for 1 hour and then vortexed at 2000 rpm for 5 min. All pellets were detached from the bottom of the tubes by hitting each tube individually on a hard surface. Then a second round of vortex was performed (2000 rpm for 5 min) and samples were centrifuged twice at 3500g for 5 min at 4°C. The barcode of each tube was scanned for sample traceability before placing it in the rack module of the Freedom EVO workstation (Tecan) integrating a vacuum system. RNA extractions were performed using NucleoSpin 96 miRNA kit (Macherey-Nagel): 600µl of clear phase were mixed with 900µl of ethanol absolute in a deep-well plate. This binding mixture was transferred to the 96 well silica membrane plate and vacuum aspiration was applied to make the RNA bind the membrane. After that, membranes were washed with 600µl of MW₁ buffer and 700µl of MW₂ buffer (X₂) with a vacuum aspiration in between. The silica membrane plate was dried (10 min, 10,000 g at RT) and RNA was eluted using 60µl of RNase-free water into 0.5 ml 2D barcoded tubes (Thermo Fisher). To avoid unnecessary RNA freeze and thaw, aliquots for quality and quantity controls were prepared and RNA samples and aliquots were frozen at -80°C until use.

20.1.2 Manual individual RNA extraction

To improve mRNA RIN or concentration for RNA-sequencing experiment, total mRNA was extracted from TruCulture cell pellet using Nucleospin miRNA kit (Macherey Nagel). The “RNA purification in combination with TRIzol lysis: small and large RNA in one fraction (total RNA)” protocol was used. TruCulture tubes were thawed, vortexed, centrifuged and 600µl of clear phase was mixed with 120µl of chloroform into a microfuge tube. The tube was vortexed, stayed 3 min at RT and centrifuged 12,000g for 15 min at 4°C. The clear phase (around 400µl) was mixed with 1ml of EtOH 100% into a new microfuge tube and transferred into a blue column for RNA binding. The column was centrifuged at 11,000g for 30 sec and washed twice with 600µl of MW1 and 700µl of MW2 buffers respectively with a centrifugation step of 11,000g for 30 sec in between. A final washing step was performed using 250µl of MW2 buffer followed by a round of centrifugation for 2 min at 11,000g. Finally, 60µl of RNase-free water was added to the column which was incubated for 1 min at room temperature. mRNA was eluted into a clean microfuge tube by centrifuging the column at 11,000g for 30 sec. mRNA was aliquoted for quality controls and stored at -80°C until use.

20.1.3 RNA quality controls

RNA concentrations were measured in 96 well plates using Quantifluor RNA system kit (Promega) following the multi-well plate quantitation high standard curve protocol. Dye was diluted 1:400 in 1X TE buffer in order to make the dye working solution. This solution was mixed and 7 standards (from 0,78 to 50 ng/ml) were prepared by serially diluting the Quantifluor RNA standard (100 ng/ml) with 1X TE buffer. After that, 200µl of dye working solution was distributed into each well of a Nunc F96 plate (ThermoFisher) and 10µl of standards, 10µl of 1X TE buffer (used as blank) and 1µl of RNA were pipetted into the plate. Plates were mixed for 5 sec on a plate shaker and incubated for 5 min at RT protected from light. Fluorescence was measured using the plate reader Tecan Infinite F200 Pro (excitation wavelength of 485 nm and emission wavelength of 535 nm). RNA concentrations were calculated by subtracting the fluorescence of the blank to all standards and RNA samples. Concentrations were extrapolated using the standard curve of fluorescence measured associated with the concentrations. Finally, obtained concentrations were multiplied by the appropriate dilution factor to get the final concentrations.

RNA integrity numbers (RIN) were defined using the Agilent RNA 6000 Nano kit (Agilent Technologies). All reagents were placed at RT at least 30 min before use. Gel was filtered and 65 μ l of it was mixed with 1 μ l of dye in a new microfuge tube. This mix was vortexed for 10 sec and centrifuged at 13000g for 10 min at RT. In the meantime, 1 μ l of RNA sample and ladder were denatured for 2 min at 70°C and stored on ice. Thereafter, 9 μ l of gel dye mix was loaded and pressurized for 30 sec in the appropriate wells. After loading 5 μ l of markers in the 13 other wells of the chip, 1 μ l of denatured RNA and ladder were distributed. The chip was vortexed for 1 min at 2000 rpm and inserted in the Agilent 2100 bioanalyzer within 5 min after its preparation. RNA quality was analyzed using the Eukaryote total RNA Nano assay of the 2100 Expert software. Samples with a RIN greater than 5 were processed for gene expression analysis.

20.1.4 mRNA quantification using Nanostring

20.1.4.1 Direct mRNA hybridization assay

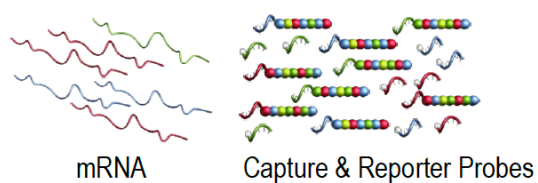
Total RNA samples were analyzed using the Human Immunology v2 panel profiling 594 immunology-related human genes including 15 internal reference genes. For every gene of the panel, a colour-coded probe pair was created. It is composed of

- A capture probe that carries a biotin molecule used for immobilization of the mRNA in a streptavidin-coated cartridge during data acquisition
- A reporter probe carrying the molecular barcode for detection and identification of the mRNA. The color codes carry 7 positions and each position can be one of 4 different colors. Thus, thousands of barcodes combinations are possible.

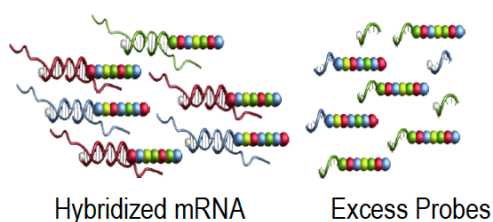
The target specific region on both the capture probe and the reporter probe are 35-50 bases long. Samples were processed twelve by twelve. Total mRNA was diluted to 20 ng/ μ l and 5 μ l was hybridized with excess of both capture and reporter probes for 16 to 30 hours at 65°C with hybridization buffer into 12-strip tubes. After the hybridization step, excess probes and non-target cellular transcripts were washed away using a two-step magnetic bead-based purification on the nCounter Prep Station. Magnetic beads derivatised with short nucleic acid sequences complementary to the capture probe and the reporter probes were used sequentially. The purified specific formed complexes were eluted and immobilized in the cartridge for data collection. Data collection was performed by the nCounter Digital Analyzer using the highest standard data resolution. 555 fields of view (FOV) were collected per flow cell with a microscope objective and a CCD camera yield-

ing data of hundreds of thousands of target molecule counts (Figure. 20.1). The barcodes were counted and tabulated in a comma separated value (CSV) format.

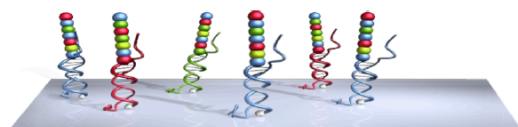
Step 1 - Hybridization



Step 2 - Purification



Step 3 - Complex immobilization



Step 4 - Image collection and barcode counting

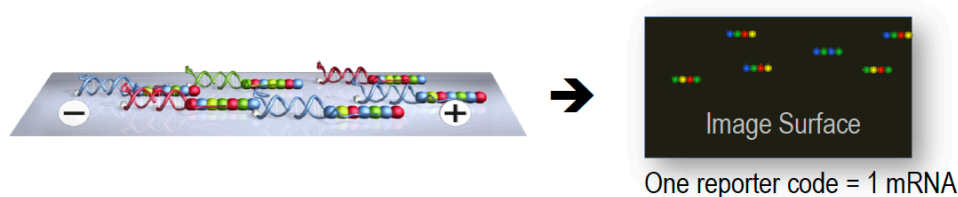


Figure 20.1: **Scientific principle of gene expression quantification using Nanostring.** Total RNA is hybridized overnight with capture and reporter probes. The magnetic bead purification step is then performed to isolate probes that have bound to targeted RNA. The capture probes of the hybridized complex bind to the surface of the cartridge coated with streptavidin and finally, the reporter probes are aligned for image collection and barcode counting.

20.1.4.2 Quality controls and data normalization

Raw Nanostring data are provided by the nCounter instrument in Reporter Code Count (RCC) file format. Each file contains the data for one sample.

- Read RCC data

To start Nanostring data pre-processing, all RCC files were imported into R using the developed “read.RCC” function. This function was developed to work for NanoString CodeSets containing 608 probes (including positive and negative control probes).

- Quality controls

Before any analysis, the quality of the data was assessed according to the Nanostring analysis guidelines. The following metrics were checked:

- Field of view counted (flag if < 0.75)
- Binding density (flag if not between 0.05 and 2.25)
- Linearity of positive controls (flag if $R_2 < 0.95$)
- Limit of detection for positive controls (flag if 0.5fM positive control < 2 standard deviations above the mean of the negative controls)
- Total counts (flag if not between 120k and 1910k)

- Probes control

Nanostring provides 6 positive and 8 negative control probes in order to control for differences between experimental variables (e.g. hybridization, purification or binding efficiency) and to detect non-specific binding (so called background level), respectively. Counts of control probes are expected to vary between samples, but independently of the stimulus. However, significant differences in counts of negative probes NEG_B and NEG_H were observed between conditions. Most probably this was due to cross-reactivity of these two probes with bacterial nucleic acid present in TruCulture stimulation systems. Thus, the negative probes NEG_B and NEG_H were removed from the analysis. Also, for all positive control probes more counts in the non-stimulated condition were observed when compared to the stimulated conditions.

This might have been due to higher expression of the targeted genes in the stimulated conditions than in the non-stimulated one, which resulted in an increased competition between positive control probes and gene probes to bind to the slide surface. More importantly, the differences in binding of positive control probes in non-stimulated and stimulated conditions were specific to the control RNA concentration (i.e., the difference in binding was the strongest for probe POS_A (128), and the weakest for probe POS_F (0.125)). As, the normalization method recommended by Nanostring does not correct for the last issue, an alternative method called “linear” was developed (based on linear regression between counts of positive control probes and the reference values).

- Gene expression data normalization

Gene expression of 25 donors of the MI cohort aged 30-39 and stratified by gender (13 women, 12 men) was measured for all stimuli with the Legacy batch [24]. This batch was also used to assess gene expression of the 1,000 donors upon Null condition. However, gene expression of the LPS and Poly(I:C) stimulation for the 975 remaining donors was measured using a different batch of CodeSet (XT batch). As gene expression of TruCulture cell pellet mRNA upon Null stimulation was determined using a different batch of Nanostring CodeSets than LPS stimulation, data normalization was performed to be able to compare the two datasets. For this, mRNA of the 25 donors upon LPS and Poly(I:C) stimulations was measured again using the XT batch as well as 25 Null mRNA samples randomly selected in the cohort across whole age span and between sexes.

Data normalization was performed in two steps. In the first step, the 75 samples run with both legacy and XT batches were used for positive control normalization and calibration between chemistries. Positive control normalization was performed using the linear method (normalization by fitting regression lines) as described in the “Probes control” part. Then, calibration factor for the XT chemistry was calculated by fitting a linear model for each gene separately. For the second step, positive control normalization using the linear method was performed for all samples that were run with the XT chemistry. Finally, differences in RNA sample input was corrected using the “local.geo.mean” method. This method calculates the geometric mean of all gene probe counts for each sample. A scaling factor for each sample was calculated as the difference between the average across the means within the conditions to which the sample belonged and the

mean of the sample. For each sample, its corresponding scaling factor was added to each gene count. This approach is based on the assumption that the total count is the same between samples within one condition, but might be different between conditions.

20.1.4.3 Differential gene expression analysis

In order 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 regress out any gene expression differences due to cell population differences between donors. The analysis was implemented using the R package “broom” vo.5.2. Multiple testing correction was then applied to select the significant genes.

20.1.5 RNA-sequencing

20.1.5.1 Experimental methods and workflow

Library preparation and sequencing were performed by Macrogen Inc. (South Korea). Libraries were prepared using TruSeq stranded total RNA with ribo zero globin kit (Library protocol: TruSeq Stranded Total RNA Sample Prep Guide, Part 15031048 Rev. E) starting with an initial quantity of 1 μ g total RNA. Cytoplasmic and mitochondrial ribosomal RNA as well as globin mRNA were removed in a single step with a bead-based depletion. RNA was then fragmented and cDNA synthesis was performed to reverse transcribe the cleaved RNA fragments. RNA template was removed during the second strand cDNA synthesis and 3' ends were then adenylated. During this step an “A” nucleotide is added to the 3' end of the fragments to prevent the cDNA strands from ligating to each other during the following step: the adapter ligation reaction. This step ligates indexing adapters to the 5' and 3' ends of the double-stranded cDNA to prepare them for the hybridization onto the sequencing flow cell. Finally, adapter-ligated fragments were PCR amplified and gel purified. cDNA was paired-end sequenced using the Novaseq Illumina system. More than 120M reads of 101bp per sample were generated and sequencing raw data files were delivered in fastq format for data analysis.

20.1.5.2 Analysis pipeline

The quality of the raw fastq files was assessed using FastQC (vo.10.1) and MultiQC (v1.7). Ribosomal RNA was removed from the forward and reverse fastq files using

the SorMeRna tool (v2.1b) and quality assessment of this pre-processing step was performed using MultiQC. To perform differential gene expression analysis, reads were aligned to the reference genome hg19 using STAR (v2.5.0a). For this, genome indexes were first generated using the genome reference sequences hg19 fasta file and the release 29 of the human comprehensive gene annotation GTF file from Gencode. Then mappings to the reference genome were run using STAR generating binary alignment map (BAM) files. Sequence alignment data in BAM format were sorted by reference coordinates and indexed using Samtools (v0.1.19). Quality assessment of the mapping step was performed using the produced log files with MultiQC. Mapped reads were counted using the featureCounts function of the subread tool (v1.6.1). The number of reads assigned to each gene were reported in a text file for each sample. In parallel, pseudoalignments to the human transcriptome were performed using post SorMeRna fastq files with Kallisto (v0.43.1) to perform differential transcript analysis. Transcriptome index was build using the release 30 of the human transcript by Gencode. Then, pseudoalignment and transcript quantification were performed with Kallisto for each sample. Alignment scores were assessed using MultiQC. Tab separated value (tsv) files were imported into R (v3.5.3) using the tximport package. In both analyses, count data transformation to stabilize the variance across the mean, visualization and normalization of the data as well as differential expression analysis were performed using the DESeq2 package in R (Figure. 20.2).

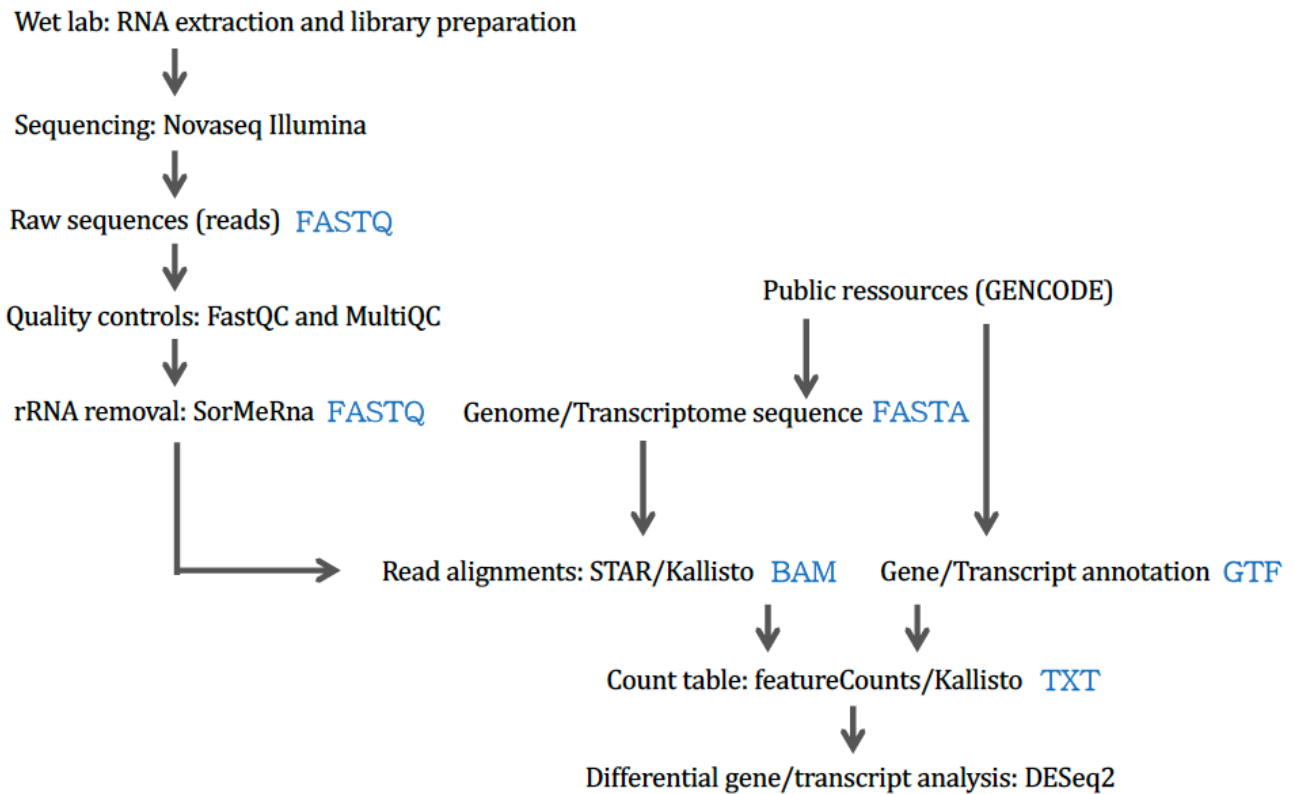


Figure 20.2: **RNA-sequencing experimental and data analysis workflow.** Total RNA was extracted from the cell pellets of TruCulture tubes and libraries were prepared for RNA-sequencing using Novaseq Illumina sequencer. Quality of the raw sequences was assessed before removing ribosomal RNA reads. Sequences were mapped to the human genome or transcriptome using STAR or Kallisto tools respectively and count tables for each sample were then imported into R for differential gene or transcript analysis using the DESeq2 package.

20.2 Protein quantification using ultrasensitive Simoa

To quantify protein cytokines at ultrasensitive concentrations, we utilized Single Molecule array (Simoa) technology. This is based on counting enzyme-labeled immunocomplexes of protein of interest captured on paramagnetic beads in single-molecule arrays. Briefly, the diluted protein is captured on magnetic beads attached with a specific antibody. The biotinylated detector antibody coupled with streptavidin- β -galactosidase also binds to the protein of interest and the immunocomplex is isolated in a reaction chamber that can only hold a single bead. Finally, β -galactosidase substrate is added and fluorescence imaging is used to detect the single protein molecule. The protein concentration is determined by calculating the ratio between the number of wells that contain a fluorescent bead and the total number of wells that contain beads (AEB: average enzymes per bead).

20.2.1 Homebrew capture antibody beads conjugation

20.2.1.1 Capture antibody buffer exchange

An initial quantity of 130µg of capture antibody was added to an Amicon Ultra 0.5ml filter (Merck Millipore) and bead conjugation buffer (Quanterix) was added to reach 500µl. The filter was centrifuged at 14,000g for 5 min and the flow-through was discarded. The filter was washed twice by adding 450µl of bead conjugation buffer followed by centrifugation at 14,000g for 5 min. The filter column was then placed into a clean microfuge tube upside down and the antibody was collected by centrifuging at 1,000g for 2 min. The membrane was washed and centrifuged twice at 1,000g for 2 min with 50µl of bead conjugation buffer. The concentration of the antibody was measured using the Nanodrop. The antibody was diluted to 0.5 mg/ml with bead conjugation buffer and stored on ice. The total volume of antibody was referred to as 2 volumes (2V) and all the following reagents volumes were adapted accordingly.

20.2.1.2 Paramagnetic beads preparation

2.8×10^8 homebrew carboxylated paramagnetic beads (1V) (Quanterix) were transferred into a microfuge tube and placed on a magnetic separator. Buffer was discarded and the tube removed from magnet. Beads were washed three times with bead wash buffer (Quanterix) (2V) and twice with bead conjugation buffer (2V). Between each wash, the tube was placed on a magnetic separator for 1 min and diluent was removed. For 1V of beads, 190µl of bead conjugation buffer was added to the beads.

20.2.1.3 Bead activation

10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific) was dissolved with 1ml of cold bead conjugation buffer. Once the solution was homogeneous, 10µl of cold diluted EDC was added to 1V of the washed beads. The solution was vortexed for 10 sec and directly placed in a shaker at 1,000 rpm for 30 min at room temperature.

20.2.1.4 Conjugation of the bead with the antibody

The tube containing the beads was placed on a magnetic separator, diluent was removed and beads were washed with 2V of bead conjugation buffer. After removing the buffer

using the magnet, the cold buffer-exchanged antibodies were quickly added to the activated beads. The tube was vortexed for 10 sec and directly placed in a shaker at 1,000 rpm for 2 hours at room temperature to keep beads in suspension.

20.2.1.5 Bead blocking and final clean-up

The tube was placed on magnetic separator for 1 min and diluent was discarded. Beads were washed twice with bead wash buffer (2V). Between each wash, the tube was placed on magnetic separator for 1 min and diluent was removed. Bead blocking buffer (Quanterix) (2V) was added to the tube that was vortexed for 5 sec and incubated in the shaker at 1,000 rpm for 30 min at room temperature. Finally, the beads were washed with 2V of bead wash buffer and with 2V bead diluent (Quanterix). Between each wash, the tube was placed on a magnetic separator for 1 min and diluent was removed. Coated and blocked beads were stored in 2V bead diluent at 4°C until use.

20.2.2 Homebrew detector antibody biotinylation

20.2.2.1 Detection antibody buffer exchange

An initial quantity of 130µg of detection antibody was added to an Amicon filter and biotinylation reaction buffer (Quanterix) was added to reach 500µl. The filter was centrifuged at 14,000g for 5 min and the flow through was discarded. The filter was washed twice by adding 450µl of biotinylation reaction buffer followed by centrifugation at 14,000g for 5 min. The filter column was then placed into a clean microfuge tube upside down and the antibody was collected by centrifuging at 1,000g for 2 min. The membrane was washed and centrifuged twice at 1,000g for 2 min with 50µl of biotinylation reaction buffer. The concentration of the antibody was measured using Nanodrop and the antibody was diluted to 1 mg/ml with biotinylation reaction buffer.

20.2.2.2 Detection antibody biotinylation

N-hydroxysuccinimide-polyethylene-glycol₄-biotin (NHS-PEG₄-Biotin) (Thermo Fisher Scientific) was placed at room temperature and resuspended with a total of 383 µL of distilled water to achieve a 3.4 mM concentration. The detection antibody was mixed with the diluted biotin at the desired ratio (40x ratio = 100µl of antibody with 3µl biotin; 60x ratio = 100µl of antibody with 4.5µl biotin). The mix was vortexed and incubated for 30 min at room temperature.

20.2.2.3 Purification of the biotinylated detection antibody

The biotinylation reaction was transferred to an Amicon filter and the biotinylated antibody was purified by performing three washing steps as described in the buffer exchange part of this method using 400, 450 and 450 μ l of biotinylation reaction buffer respectively. The antibody was collected and the membrane was washed twice with 50 μ l of biotinylation reaction buffer. The volume and concentration of the purified biotinylated detection antibody was assessed and the tube was stored at 4°C until use.

20.2.3 Simoa homebrew assay

All homebrew assays (Table. 20.1) were performed using a 2-step configuration in which capture and detection antibodies are incubated simultaneously with the diluted plasma/sample.

20.2.3.1 Reagents and samples preparation

The number of measurements (n) was calculated by adding the number of sample measures to the number of standard measures used for the calibration curve. The volume of diluted beads to declare to the machine was calculated by multiplying n to 35 μ l (volume of capture beads used per measurement including dead volume). The volume of diluted beads to prepare was calculated by adding 600 μ l to the volume of beads to declare. The volume of beads to use was calculated in order to reach 12 μ l of beads per ml of volume to prepare. Beads were washed with homebrew detector/sample diluent (Quanterix) (12.5 μ l of diluent/ μ l of beads) in a 1.5ml microfuge tube. The tube was placed on magnetic separator for 1 min and diluent was discarded. Beads were then diluted into the calculated volume of homebrew detector/sample diluent (volume to prepare). The volume of detector to declare to the machine was calculated by multiplying n to 30 μ l (volume of biotinylated detector antibodies used per measurement including dead volume) and the volume of detector to prepare was calculated by adding 600 μ l to the volume of detector to declare. Detector was diluted into homebrew detector/sample diluent to reach the optimized concentration. The enzyme conjugate streptavidin- β -galactosidase (SBG) was diluted to 50pM or 150pM using SBG diluent (Quanterix). Standards were diluted with homebrew detector/sample diluent according to the developed assay. Plasma supernatants were centrifuged at 10,000 rpm at 4°C for 15 min and distributed in a 96 well plate with a dilution factor of minimum 1:3 into homebrew detector/sample diluent.

When performing the IFN β assay, standards and samples were prepared using Homebrew sample diluent B (Quanterix) to reduce non-specific interactions observed when using the regular homebrew detector/sample diluent.

20.2.3.2 Simoa sample processing and analysis

Diluted beads, detector and SBG were loaded and the appropriate volume to declare was notified to the machine. Then, fluorogenic enzyme substrate, resorufin- β -D-galactopyranoside (RGP) bottles (Quanterix) were loaded (one bottle per 96 measures). Finally, 96 well plates containing the standards and samples were loaded and the appropriate homebrew protocol was associated to each well that required to be analyzed. After the run, the AEB data for each sample were exported and samples concentration extrapolation as well as the LoD were determined using GraphPad Prism 8.3.0.

Homebrew assay	Capture antibody	Detector antibody	Standard	Optimized conditions	Limit of detection
IL-12p70	BD ref. 555065	BD ref. 554660	R&D ref. 219-IL-005	2 steps Detector 0,3 µg/ml SBG 150pM	0.01 pg/ml
IL-23	eBiosciences ref. 14-7238	BD ref. 554660	R&D ref. 1290-IL-010	2 steps Detector 0,3 µg/ml SBG 150pM	0.04 pg/ml
IL-12p40	BD ref. 551227	BD ref. 554660	R&D ref. 309-IL-002	2 steps Detector SBG 150pM	0.07 pg/ml
IL-17A	Biologend ref. 512702	Mabtech ref. 35206	R&D ref. 317-ILB	2 steps Detector 0,3 µg/ml SBG 150pM	0.04 pg/ml
IL-17F	ImmunoQure 17E3	ImmunoQure 9A2	R&D ref. 1335-IL/CF	2 steps Detector 0,5 µg/ml SBG 50pM	0.0006 pg/ml
IL-22	ImmunoQure 51G4	ImmunoQure 30G1	R&D ref. 782-IL/CF		2.4 pg/ml
Pan-alpha	ImmunoQure 8H1	ImmunoQure 12H5	PBL ref. 11150	2 steps Detector 0,3 µg/ml SBG 150pM	0.05 fg/ml
IFNβ	PBL ref. 710322-9	PBL ref. 710323-9	PBL ref. 11415-1	2 steps Detector 1 µg/ml SBG 50pM	0.07 pg/ml
IFNγ	Biologend ref. 507501	R&D ref. MAB285	PBL ref. 11500	2 steps Detector 0,3 µg/ml SBG 150pM	0.005 pg/ml

Table 20.1: **Homebrew Simoa assays antibodies, standards and running condition details.**

21. Healthy human fresh whole blood

To test specific hypotheses generated from *Milieu Interieur* results, we recruited additional healthy donors for experimental validation. Relevant information regarding how this data was generated and analyzed is described

21.1 Recruitment

For intracellular flow cytometry staining, isolation of cell populations and identification of low IL-12p70 responders, fresh blood was collected into sodium heparin tubes from healthy donors enrolled at the Clinical Investigation and Access to BioResources platform (ICAReB) at the Institut Pasteur. These donors are part of the CoSImmGen cohort. The biobank activity of this platform is certified ISO 9001 and NFS 96-900. Written informed consent was obtained from all study participants.

21.2 Whole blood stimulation assays

To mimic the whole blood stimulation performed with TruCulture tubes, fresh whole blood was diluted 1:3 with RPMI medium 1640 (1X) + GlutaMAX into 5ml Polystyrene round-bottom tube. Diluted whole blood was either left without any stimulant to mimic the Null condition or stimulated with LPS (LPS-EB ultrapure InvivoGen) at the final concentration of 10 ng/ml, Poly(I:C) (HMW VacciGrade InvivoGen) at the final concentration of 20 µg/ml, R848 (Vaccigrade InvivoGen) at the final concentration of 1µM, IFN α (IntronA interferon alpha-2b Shering-Plough) at the final concentration of 1000 IU/ml, IFN β (Betaferon interferon beta-1b Bayer) at the final concentration of 1000 IU/ml, IFNAR1 blocking antibody (Abcam) at the final concentration of 15 µg/ml or IFNAR2 neutralizing antibody (PBL clone MMHAR-2) at the final concentration of 15 µg/ml. Tubes were vortexed and incubated at 37°C, 5% CO₂ for the required time.

21.3 Flow cytometry intracellular cytokine staining

Fresh whole blood of healthy donors was collected from healthy volunteers recruited at the Institut Pasteur ICAReB Platform. Blood was diluted 1:3 with RPMI + GlutaMAX (Gibco, Life Technologies) without any stimulant (null condition) or with LPS at the final concentration of 10 ng/ml (LPS-EB ultrapure InvivoGen). Diluted blood was vortexed and incubated at 37°C and brefeldin A (GolgiPlug, BD Biosciences) was added 8 hours after the beginning of the stimulation (diluted 1:1000). After 22 hours of stimulation, whole blood was centrifuged at 500g for 5 min at room temperature (RT) and supernatant was removed. 2mM final concentration EDTA was added to each tube and samples were placed at 37°C for 10 min in order to detach cells from the tubes. Red blood cells were lysed during 10 min on ice using Pharm Lyse solution (BD Biosciences). Tubes were centrifuged 500g for 5 min at 4°C and supernatants were discarded. Leukocytes were washed with PBS 1X (Gibco, Life Technologies), centrifuged 500g for 5 min at 4°C and, after removing the supernatant, cells were resuspended into fresh PBS. Live dead fixable green (Thermo Fisher Scientific) was added 1:1000 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) followed by a centrifugation at 500g for 5 min at 4°C. FcR blocking reagent (Miltenyi) was added to the resuspended cells followed by 10 min incubation at 4°C in the dark. Surface antibody premix to assess IL-12p70 and IL-23, IFN β or IFN γ secretion (Tables. 21.1, 21.2 and 21.3 respectively) was prepared and distributed to each sample. Cells were incubated in the dark for 30 min at 4°C. Thereafter, cold PBS was added to the tubes which were centrifuged at 500g for 5 min at 4°C and the supernatant was removed. Cells were resuspended and 250 μ l of Fixation/Permeabilization solution (Cytofix/Cytoperm, BD Biosciences) was added to each tube and samples were incubated at 4°C for 15 min protected from light. After that, cells were washed with cold PBS followed by centrifugation at 500g for 5 min at 4°C and supernatant was discarded. Cells were resuspended, washed in 1ml of 1X Perm/Wash buffer (Cytofix/Cytoperm, BD Biosciences) and centrifuged at 500g for 5 min at 4°C. Supernatant was removed and 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. IL-12p70 and IL-23 antibodies were added with 1X Perm/Wash buffer (100 μ l 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 in cold PBS and centrifuged at 500g for 5

min at 4°C. Supernatant was removed and samples were resuspended in 600µl of 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 (gating strategy described in (Figure. 21.1)). Statistical graphs were done using Prism 8.

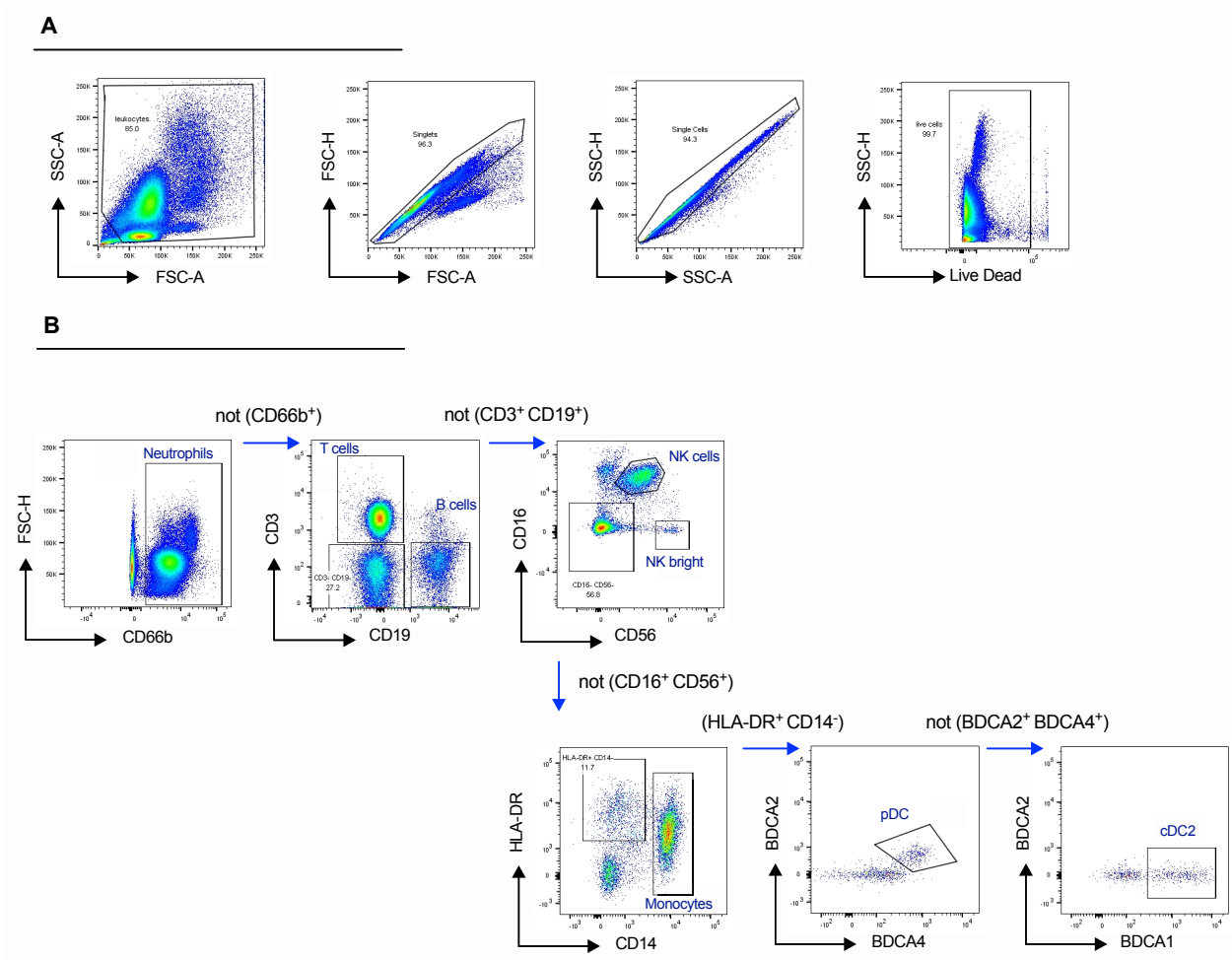


Figure 21.1: **Flow cytometry gating strategy used to isolate immune cell populations.** (A) Live leukocytes were first identified. Doublets were excluded using forward scatter height (FSC-H) and forward scatter width (FSC-A) followed by side scatter height (SSC-H) and side scatter area (SSC-A). Dead cells were removed using a live/dead marker. (B) Expression of CD66b was used to identify neutrophils. T cells and B cells were identified within the CD66b⁻ cells based on CD3 and CD19 expression respectively. Within the CD66b⁻, CD19⁻ and CD3⁻ cells, NK CD56⁺ CD16⁺ cells and NK bright cells CD56⁺ CD16⁻ were identified. Within the CD66b⁻, CD19⁻, CD3⁻, CD56⁻, CD16⁺ cells, monocytes were gated based on the expression of CD14 and dendritic cells were identified among HLA-DR⁺, CD14⁻ cells. pDCs were analyzed for their expression of BDCA-2 and BDCA-4 markers whereas cDC2 were analyzed based on BDCA-1 expression.

Marker	Color	Clone	Company	Reference	Dilution used (/100µl of blood)
CD3	APC-H7	SK7	BD	560176	1/40
CD19	BV711	SJ25C1	BD	563036	1/40
CD56	Pe-Cy7	B159	BD	557747	1/20
CD14	V500	M5E2	BD	561391	1/20
CD16	PE-CF594	3G8	BD	562293	1/40
CD66b	BV421	G10F5	BD	562940	1/50
HLA-DR	PerCP-Cy5.5	L243	BD	339216	1/10
BDCA1 (CD1c)	BUV395	F10/21A3	BD	742751	1/20
BDCA2 (CD303)	AF700	201A	BioLegend	354228	1/40
BDCA4 (CD304)	BV605	U21-1283	BD	743130	1/20
IL-12p70	APC	REA123	Miltenyi	130-103-673	1/40
IL-23p19	PE	23dcdp	eBioscience	12-7823-42	1/40

Table 21.1: IL-12p70 and IL-23 intracellular staining panel.

Marker	Color	Clone	Company	Reference	Dilution used (/100µl of blood)
CD3	APC-H7	SK7	BD	560176	1/40
CD19	BV711	SJ25C1	BD	563036	1/40
CD56	Pe-Cy7	B159	BD	557747	1/20
CD14	V500	M5E2	BD	561391	1/20
CD16	PE-CF594	3G8	BD	562293	1/40
CD66b	BV421	G10F5	BD	562940	1/50
HLA-DR	PerCP-Cy5.5	L243	BD	339216	1/10
BDCA1 (CD1c)	BUV395	F10/21A3	BD	742751	1/20
BDCA2 (CD303)	AF700	201A	BioLegend	354228	1/40
BDCA4 (CD304)	BV605	U21-1283	BD	743130	1/20
IL-12Rβ1	PE	69310	R&D systems	FAB839P	1/20
IFNγ	FITC	B27	BioLegend	506504	1/40

Table 21.2: IFNγ intracellular staining panel.

Marker	Color	Clone	Company	Reference	Dilution used (/100µl of blood)
CD3	APC-H7	SK7	BD	560176	1/40
CD19	BV711	SJ25C1	BD	563036	1/40
CD56	Pe-Cy7	B159	BD	557747	1/20
CD14	V500	M5E2	BD	561391	1/20
CD16	PE-CF594	3G8	BD	562293	1/40
CD66b	BV421	G10F5	BD	562940	1/50
HLA-DR	PerCP-Cy5.5	L243	BD	339216	1/10
BDCA1 (CD1c)	BUV395	F10/21A3	BD	742751	1/20
BDCA2 (CD303)	AF700	201A	BioLegend	354228	1/40
BDCA4 (CD304)	BV605	U21-1283	BD	743130	1/20
IFNβ	FITC	MMHB-3	PBL	21400-3	1/40

Table 21.3: IFNβ intracellular staining panel.

21.4 Time series experiment and analysis

Whole blood of 5 healthy donors was stimulated into 14 TruCulture tubes during 14 timepoints (0-30 hours) with null and LPS stimulants. RNA was extracted from the cell pellet and gene expression measured using Nanostring. In parallel, protein secretion was measured in the supernatants using Simoa. 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 predictors, and the donors were modelled as a random effect. The model was implemented using the R package nlme v3.1.140.

21.5 Linear regression model to predict IFN β and IL-12p70 secretion

Variable selection was performed using the feature selection algorithm Boruta package of R [237]. All selected variables were tested for multicollinearity by computing the variance inflation factor using the caret package in R. IFN β and IL-12p70 selected variables were included in a linear regression model. To confirm that the model was appropriate for the data, patterns of residuals diagnostic plots were checked. Finally, the proportion of the variance explained by each category of variable was determined by averaging the sums of squares in all orderings of the variables in the linear model using the lmg metric in the relaimpo package of R [289].

22. Disease cohorts

22.1 Hepatitis C cohort

Patients infected with hepatitis C virus were recruited by four different hospitals/centers in Paris Ile-de-France. The cohort included 100 donors from 18 to 70 year old infected with the genotype 1 or 4 of the virus. Blood of the hepatitis C infected patients was collected prior to interferon treatment initiation. All patients were treated for 6 months with a combination of pegylated interferon and ribavirin which was the standard of care therapy at the time. Written informed consent was obtained from all study participants.

22.2 Tuberculosis cohort

Patients with Tuberculosis disease were recruited by the South African Tuberculosis Vaccine Initiative (SATVI) in Worcester, South Africa. The cohort included 24 individuals with active TB disease and 24 latently *Mycobacterium tuberculosis* infected patients. Age and sex of the latent group was comparable to the active TB group. Blood of the TB active patients was collected prior to treatment initiation. The TB clinical study protocols and informed consent forms were approved by the Human Research Ethics Committee of the University of Cape Town (ref: 234/2015).

Part VII

Appendices

23. Abstracts of co-authored studies in preparation for submission

23.1 Tuberculosis impacts immune-metabolic pathways resulting in perturbed IL-1 cytokine responses

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Summary:

Tuberculosis (TB) remains a major public health problem. Host-directed therapeutics are proposed as novel treatment strategies. However, their successful development still requires a comprehensive understanding of how *Mycobacterium tuberculosis* (*M.tb*) infection impacts immune and metabolic responses. To address this challenge, we applied standardised immunomonitoring tools to compare induced immune responses between individuals with latent *M.tb* infection (LTBI) and active TB disease. This revealed distinct responses between TB and LTBI groups at transcriptomic, proteomic and metabolomic levels. We observed dysregulated IL-1 responses to BCG in TB patients, which could be explained through differences in granzyme B and TNF α . At baseline, we identified pregnane steroids and the PPAR γ pathway as novel immune-metabolic drivers of IL-1ra secretion. These findings improve our knowledge of how *M.tb* alters immune responses, and may support design of improved diagnostic, prophylactic and therapeutic tools.

23.2 Systems biology demonstrates the predominant role of circulating interferon-alpha in primary Sjögren's syndrome and a genetic association with the class II HLA DQ locus

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23.2. Systems biology demonstrates the predominant role of circulating interferon-alpha in primary Sjögren's syndrome and a genetic association with the class II HLA DQ locus

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Summary:

Primary Sjögren's syndrome (pSS) is the second most frequent systemic autoimmune disease affecting 0.1% of the general population. No specific immunomodulatory drug has demonstrated efficacy for this disease, and no biomarker is available to identify patients at risk of developing systemic complications. To support a precision medicine approach, we integrated transcriptomic, proteomic, cellular and genetic data sets with clinical phenotypes in a cohort of 351 pSS patients. Transcriptomic analysis showed a strong IFN gene signature that enabled patient stratification matching clinical assessments, and was replicated in three independent cohorts. As gene expression analysis did not discriminate between type I and II interferons, we applied digital ELISA to demonstrate that the IFN transcriptomic signature was driven by circulating IFN α protein levels. This cytokine, detectable in 75% of patients, was significantly associated with clinical and immunological features of disease activity at enrollment, and with more frequent systemic complications during the 5-year follow-up. Genetic analysis revealed a significant association between IFN α protein levels and a MHC-II HLA-DQ locus and anti-SSA antibody. Additional cellular analysis revealed that the polymorphism acts through upregulation of HLA II molecules on conventional DCs. Therefore, our systems biology approach enabled patient stratification and identified the role of IFN α in pSS through an association with HLA gene polymorphisms that results in systemic complications.

24. **Published co-authored studies**



Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges

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The contribution of host genetic and nongenetic factors to immunological differences in humans remains largely undefined. Here, we generated bacterial-, fungal-, and viral-induced immune transcriptional profiles in an age- and sex-balanced cohort of 1,000 healthy individuals and searched for the determinants of immune response variation. We found that age and sex affected the transcriptional response of most immune-related genes, with age effects being more stimulus-specific relative to sex effects, which were largely shared across conditions. Although specific cell populations mediated the effects of age and sex on gene expression, including CD8⁺ T cells for age and CD4⁺ T cells and monocytes for sex, we detected a direct effect of these intrinsic factors for the majority of immune genes. The mapping of expression quantitative trait loci (eQTLs) revealed that genetic factors had a stronger effect on immune gene regulation than age and sex, yet they affected a smaller number of genes. Importantly, we identified numerous genetic variants that manifested their regulatory effects exclusively on immune stimulation, including a *Candida albicans*-specific master regulator at the *CR1* locus. These response eQTLs were enriched in disease-associated variants, particularly for autoimmune and inflammatory disorders, indicating that differences in disease risk may result from regulatory variants exerting their effects only in the presence of immune stress. Together, this study quantifies the respective effects of age, sex, genetics, and cellular heterogeneity on the interindividual variability of immune responses and constitutes a valuable resource for further exploration in the context of different infection risks or disease outcomes.

human immune variation | gene expression | genetics | sex | age

Unraveling the contributions of host and environmental factors to interindividual variability in immune responses is crucial to understand immune pathology (1). Immunological research has largely neglected the concept of interindividual heterogeneity, but there is now growing biomedical interest in studies of the variation of the immune response and its determinants in healthy populations (2)—a strategy known as systems or population immunology (1, 3, 4). Recent cohort-based studies have shed light on how host genetic and nongenetic factors, including environmental variables (e.g., annual seasonality, nutrition, latent infections) and variation of the commensal microbiota, drive the plasticity of immune responses. For example, intrinsic factors, such as age and sex, have an impact on cellular and molecular phenotypes, such as immune cell and protein levels (5–12), and genetic variants also account for a significant fraction of the observed variation of these immune traits (5, 6, 8, 13–16).

In terms of gene expression, immune responses vary markedly between individuals and populations (17–22), but the extent of this variation and its drivers are only beginning to be clarified (1, 3, 23). Recent whole-blood studies have shown that age and sex strongly affect gene expression in the basal state (12, 24, 25). Likewise, genetic variation is an important source of variability in gene expression (20, 26–28). The mapping of expression quantitative trait loci (eQTLs; genetic variants that affect gene expression variation) has become an important approach in translational medicine (29), as regulatory variants are increasingly recognized as contributing to complex disease risk (22, 23, 26–28, 30, 31). eQTLs are particularly informative in studies of immune phenotypes,

Significance

Identifying the drivers of the interindividual diversity of the human immune system is crucial to understand their consequences on immune-mediated diseases. By examining the transcriptional responses of 1,000 individuals to various microbial challenges, we show that age and sex influence the expression of many immune-related genes, but their effects are overall moderate, whereas genetic factors affect a smaller gene set but with a stronger effect. We identify numerous genetic variants that affect transcriptional variation on infection, many of which are associated with autoimmune or inflammatory disorders. These results enable additional exploration of the role of regulatory variants in the pathogenesis of immune-related diseases and improve our understanding of the respective effects of age, sex, and genetics on immune response variation.

Author contributions: B.P., D.D., M.L.A., and L.Q.-M. designed research; B.P., A.U., H.Q., C.P., B.C., M.H., B.A., D.G., and J.F. performed research; M.I.C. contributed conceptual and analytic tools; B.P., D.D., E.P., J.B., V.R., C.R.M., M.L.A., and L.Q.-M. analyzed data; and B.P., D.D., E.P., M.L.A., and L.Q.-M. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The genotype data reported in this paper have been deposited in the European Genome-Phenome Archive (EGA; accession no. EGAS00001002460).

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in which interactions between genetic and environmental factors, such as infection, may be required for phenotypic manifestations (23, 32). In this context, thousands of eQTLs that only appear after immune challenge (i.e., response eQTLs) have been identified over the last years (17–19, 21, 22, 33, 34), establishing putative functional links between expression phenotypes and organismal traits, such as immunity to infection (23, 26, 32). Furthermore, recent data suggest that immune-related response eQTLs play an important role in the genetic architecture of human diseases (35).

Despite the major contribution of systems immunology studies to the increased comprehension of human immune system variation (4), important questions remain to be systematically explored. The investigation of how intrinsic factors impact gene expression variation on infection is missing, yet this is critical to understand the observed inequalities among individuals of different ages and sexes in immune responses and disease risk (36, 37). Furthermore, most studies have focused on isolated cell types treated with single agonists and have not quantified jointly the influence of the genetic and nongenetic drivers of gene expression variation on immune stimulation or infection in a multicellular environment.

In this study, we adopted an integrative approach, combining genetic, transcriptomic, and cytometric data. We generated 7,000 immune transcriptional profiles for whole-blood samples, after stimulation with a wide range of microbes, from 1,000 healthy individuals of European ancestry stratified by age (20–69 y old, 200 per decade) and sex (500 women, 500 men). This balanced experimental design (Fig. S1) provided a unique opportunity to delineate the respective effects of age, sex, and genetic factors and of inherent variation in immune cell populations on the interindividual variability of immune responses to infection. In doing so, our study lays the foundations for future precision medicine clinical strategies that may stratify patient groups based on age, sex, or genetic background.

Results

Distinctive Transcriptional Responses to Bacterial, Fungal, and Viral Challenges. We stimulated whole blood with three bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus Calmette-Guérin* (BCG); a fungus, *Candida albicans*; a live virus, influenza A virus (IAV); and a superantigen, staphylococcal enterotoxin B (SEB) (Fig. S1). To limit the burden of multiple testing, we quantified the expression of 560 immune-related genes before and after immune stimulation in the 1,000-donor cohort using NanoString hybridization arrays, which produce highly reproducible transcriptional data (38). Furthermore, we measured the proportions of eight major immune cell types (i.e., neutrophils, CD19⁺ B cells, CD4⁺ T cells, CD8⁺ T cells, CD4⁺CD8⁺ T cells, CD4⁺CD8⁻ T cells, natural killer (NK) cells, and CD14⁺ monocytes) in all individuals by standardized flow cytometry.

Immune stimulation altered the expression of 87–92% of the genes tested (Dataset S1), with most genes being up-regulated [paired *t* test; false discovery rate (FDR) < 0.01] (Fig. 1A and Dataset S2). The direction of expression changes was globally shared across stimuli, with IAV inducing the most distinctive response (Fig. 1B). Principal component analysis confirmed these observations (Fig. 1C); principal component 1 (PC1) was driven mainly by genes induced by all stimuli other than IAV, reflecting a bacterial signature, whereas PC2 was driven by IAV-induced genes, reflecting a strong type 1 IFN signature (Fig. S2A and B). PC2 further distinguished *C. albicans*-induced genes from those stimulated by *E. coli* and SEB, whereas PC3 separated the genes induced by *E. coli* from those stimulated by *C. albicans* and SEB (Fig. 1C and Fig. S2C). Thus, all stimuli, except BCG and *S. aureus*, triggered distinguishable transcriptional responses, the largest differences being observed between viral and bacterial/fungal challenges.

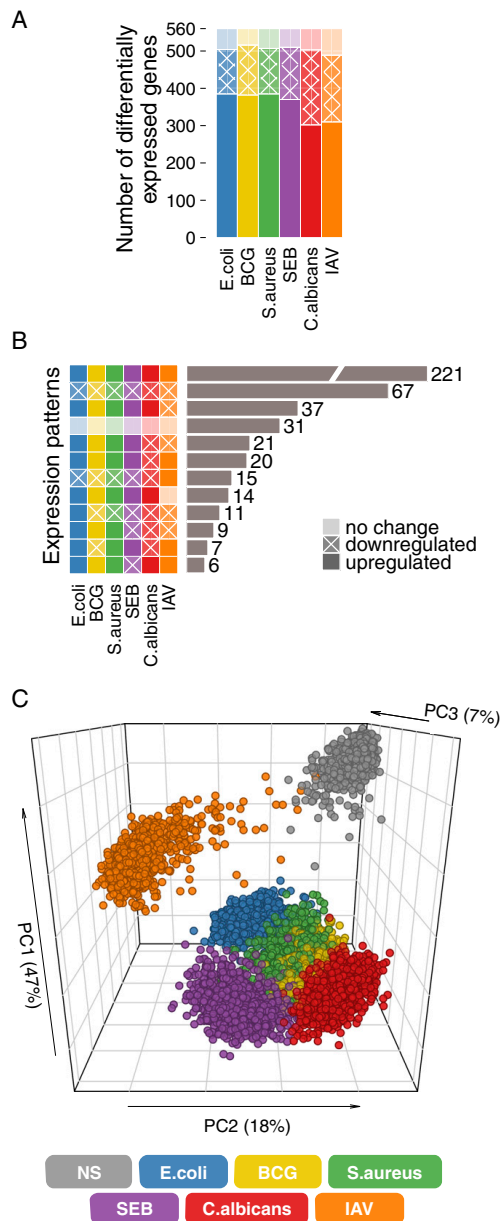


Fig. 1. Distinct transcriptional responses to bacterial, viral, and fungal infections. (A) Number of genes presenting differential expression on immune stimulation. (B) Number of genes presenting common patterns of expression changes across stimulation conditions. Only expression patterns common to at least five genes are presented. (C) Principal component analysis of immune gene expression profiles in the nonstimulated state and on immune stimulation. NS, nonstimulated control.

Widespread Effects of Age and Sex on Gene Expression Variation on Immune Stimulation. We investigated the effect of age on transcriptional responses to immune activation. For each gene, in each condition, we regressed its expression on age, while adjusting for sex, blood cell composition, and technical variables. The expression of 473 genes was linearly correlated with age in at least one condition (FDR < 0.01) (Dataset S3 and Table S1), with 267 being affected by age only on immune stimulation, highlighting the importance of examining induced transcriptional profiles. Unlike previous reports of increased gene expression variance with age in different species (39–41), we found no statistical support for age-dependent changes in expression variance in our age-balanced cohort (Breusch–Pagan test; FDR < 0.01).

To test for nonlinear associations between gene expression and age, we used ANOVA and stratified the cohort into five age groups. The results obtained were similar to those for the regression analysis for all conditions, except for IAV (Fig. 2A). The 20- to 29-y-old age group displayed a response to IAV that was different from all other groups, this difference being most marked relative to the 30- to 39-y-old age group (Fig. 2B). The relevance of the age groups selected was confirmed by testing alternative age stratification strategies (SI Materials and Methods and Fig. S3). We found 87 and 119 genes displaying increased and decreased expression, respectively, in the 20- to 29-y-old age group compared with 30- to 39-y-old individuals (Tukey Honest Significant Differences test; $P < 0.05$) (Fig. 2B and Dataset S4). Genes with increased expression were enriched in functions relating to innate immune responses as annotated in the innateDB database ($P = 6.5 \times 10^{-3}$; e.g., type 1 IFNs), whereas genes with decreased expression included genes with known roles in antibody-associated responses (e.g., *FCGRT*, *CR1*). Interestingly, the detected age differences echo a recent study that reported similar gene expression differences in H1N1 vaccine recipients under the age of 35 y old (42).

We then explored the stimulus specificity of age effects for each of the 467 genes presenting age-dependent expression (FDR < 0.01, except for IAV because of the nonlinear effect). We found that the effect of age was often stimulus-specific, with 40% of genes presenting age-dependent expression in only one or two conditions (Fig. 2C and Dataset S3). An example is shown for SEB stimulation, which displayed increased *IL-13* expression and a parallel decrease in *IL-4R* (IL-13 receptor) expression as a function of age (Fig. 2D).

As age is associated with increased pathogen infection, in particular with CMV that is known to have a broad influence on immune variation (5), we tested whether CMV seropositivity could explain the detected age-dependent expression changes. When comparing the proportions of variance explained by age in two linear regression models with and without CMV as an independent variable, no significant differences were detected between the models (Fig. S4). Although we cannot rule out that infection with other pathogens could mediate, at least partially, some age-specific effects, our results indicate that infection with CMV does not explain the widespread effects of age on microbial-induced gene expression.

Next, we investigated the influence of sex on immune response variation by regressing gene expression on sex, while adjusting for age, blood cell heterogeneity, and technical variables. We found 509 genes with expression that was sex-dependent in at least one condition (FDR < 0.01) (Dataset S3 and Table S1), with 181 being affected by sex only after stimulation. More genes displayed higher expression in women than in men across all conditions, this difference being significant for BCG, *S. aureus*, SEB, and *C. albicans* (test for one proportion; $P < 0.01$). Furthermore, 33 genes displayed differences in expression variance between the sexes in at least one condition (Levene test; FDR < 0.01) (Dataset S5). When assessing the stimulus specificity of sex effects, we found that these were often shared across stimuli; 45% of genes presented sex-specific differences in expres-

sion in more than six conditions (Fig. 2E and Dataset S3). Two key examples are *CD14* (encoding the LPS coreceptor), which was more strongly expressed in men, and *ICOS* (encoding CD278, which down-regulates T-cell activation), which was more strongly expressed in women, for all stimuli (Fig. 2F).

Together, our findings suggest an important contribution of age and sex to immune response variation, with the effects of age on transcriptional variability being highly dependent on the infectious stimulus used and those of sex being largely shared across microbial challenges.

Unraveling the Direct and Indirect Effects of Age and Sex on Gene Expression. As both age and sex influence blood cell composition (43), we next investigated whether their effects on gene expression are mediated by the eight major cell populations studied using structural equation modeling (44). For each gene with an age-related expression pattern, we built a model with eight indirect effects (one for each cell type) and one direct effect of age, while adjusting for sex, the genetic factors identified below, and technical variables (Fig. S5A). An analogous model was built for sex (Fig. S5B). The outcome of these models is such that, for any given gene, direct and/or indirect effects of age and sex may be observed simultaneously.

We found that, in the absence of stimulation, the expression of 85% of genes was directly affected by age (Fig. 3A and B and Dataset S6) and that this was the only (i.e., total) effect observed for 53% of genes (Fig. 3B). The expressions of 44 and 17% of genes were influenced by age through a decrease with age in the proportions of CD8⁺ and CD4⁺CD8⁻ T cells, respectively (the mediation of the other cell types was not statistically significant). These indirect, cell-mediated effects were often coupled with direct effects of age (Fig. 3B). Thus, only for a very small number of genes could the total effect of age on expression be explained by cellular composition. On immune stimulation, age affected expression through the same cell populations, but the CD8⁺ T-cell mediation was generally less marked than in the absence of stimulation (Fig. 3B).

Similarly, the expression of 76% of genes was directly affected by sex in the absence of stimulation (Fig. 3A and C and Dataset S7), this being the only effect observed for 55% of genes (Fig. 3C). The expressions of 26 and 21% of genes were indirectly affected by sex because of a decrease in the proportion of CD4⁺ T cells in men and a decrease in monocytes in women, respectively. After stimulation, sex effects were also mediated by CD4⁺ T cells and monocytes, and the mediation effect of monocytes was observed for a larger proportion of genes (29–49%) compared with nonstimulated cells (21%), reflecting that many of our stimuli activate monocytes.

Overall, although specific cell populations can mediate the effects of age and sex on immune-related gene expression, our analyses detected a direct effect of these intrinsic factors for the majority of immune genes affected, suggesting that age and sex effects are often mediated by mechanisms other than those affecting blood cell heterogeneity.

Mapping the Genetic Basis of Transcriptional Responses to Microbial Challenges. To investigate the contribution of genetic factors to immune gene expression variation, we mapped eQTLs through tests for associations between genome-wide SNPs (minor allele frequency >0.05) and 560 expression traits. Individuals were genotyped using the HumanOmniExpress and the HumanExome BeadChips that, after imputation using the 1,000 Genomes reference panel (45), yielded a final dataset of 5,265,361 SNPs. We used a linear mixed model to account for possible relatedness and population structure in our cohort, which was further adjusted for age, sex, blood cell heterogeneity, and technical variables.

We first mapped local, likely *cis*-acting eQTLs (within 1 Mb of each gene) and detected 239 genes presenting an eQTL in at least one condition ($P < 2.3 \times 10^{-8}$, Bonferroni-adjusted $P < 0.05$)

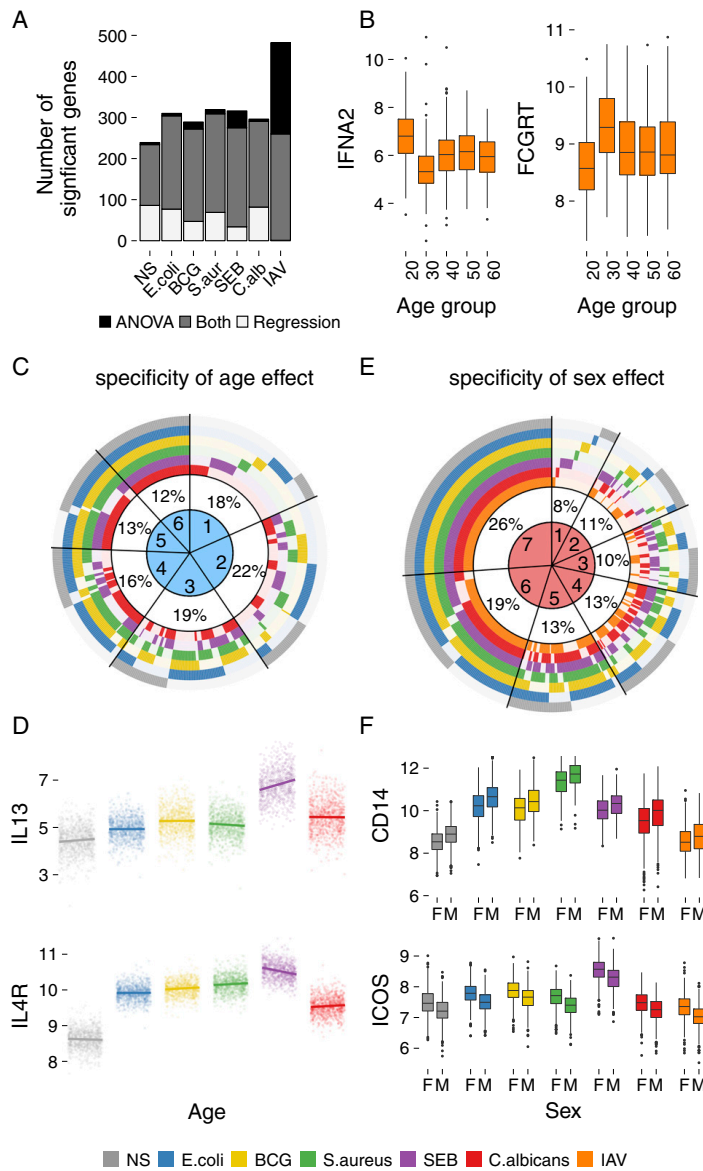


Fig. 2. Effects of age and sex on the variation of gene expression. (A) Number of genes presenting age-dependent expression changes, as detected by linear regression and ANOVA, in the absence of stimulation and after stimulation. (B) Expression patterns of *IFNA2* and *FCGR2* in response to IAV stimulation across five decades of life. (C) Specificity of age effects on gene expression across conditions. Numbers in the circle sectors (1–6) denote the numbers of stimuli for which the expression of the corresponding genes was age-dependent. The IAV condition was not considered, as it presented a nonlinear association with age. (D) Age-specific (20–69 y) expression of *IL13* and *IL4R* for each stimuli. A significant age association was observed in response to SEB stimulation. (E) Specificity of the effect of sex on gene expression across conditions. Numbers in the circle sectors (1–7) denote the numbers of stimuli for which the expression of the corresponding genes was sex-dependent. (F) Expression differences between men and women for *CD14* and *ICOS*, common to all conditions. Gene expression is presented as normalized gene counts. The legend with color-coding applies to C–F. F, female; M, male; NS, non-stimulated control.

(Fig. 4, Datasets S8–S14, and Table S2). A large proportion of eQTLs was either detected in all conditions (28%) or specific to a single condition (27%) (Fig. 5A) as illustrated by the shared eQTLs at *CTSC* and *IFI2* and the condition-specific eQTLs at *TRAF4*, *IL7R*, and *TLR3* (Fig. 5B and C). Notably, the latter cases represent response eQTLs, of which a total of 104 were identified, indicating gene–environment (G × E) interactions.

We then searched for master regulatory regions by mapping *trans*-eQTLs (i.e., variants regulating the expression of distant genes or gene networks; >1 Mb away from the transcriptional start/end site). We further verified that the detected *trans*-effects of each eQTL did not result from heterogeneity in immune cell subpopulations (*SI Materials and Methods*). In the absence of stimulation, we identified four *trans*-eQTLs ($P < 1.7 \times 10^{-11}$,

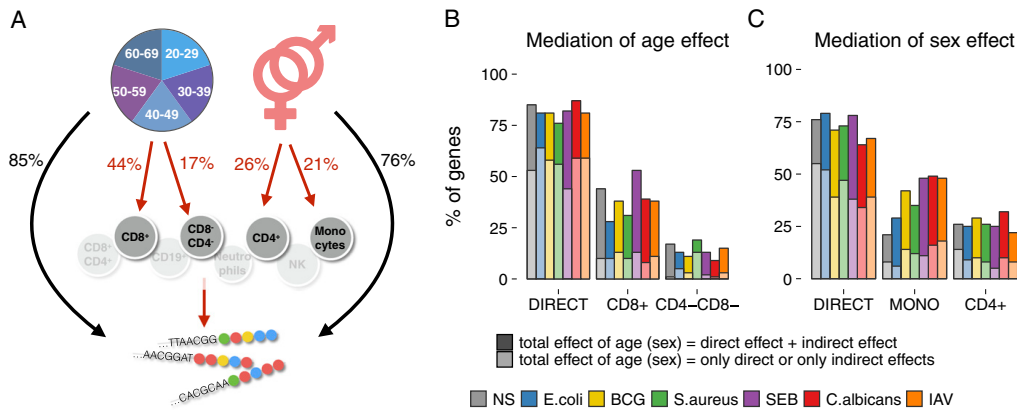


Fig. 3. Structural equation models. (A) Mediation of the effects of age and sex on gene expression by blood cell composition in the nonstimulated control. (B) Fraction of genes displaying expression directly or indirectly affected by age across stimulation conditions. (C) Fraction of genes displaying expression directly or indirectly affected by sex across conditions. NS, nonstimulated control.

Bonferroni-corrected $P < 0.05$), the strongest of which was located in the *FCGR3A/HSPA6* region and regulated *GBP5*, *STAT1*, *FCGR1A*, *GBP1*, and *IRF1*. We found that *trans*-regulation had a stronger effect after stimulation, particularly with *E. coli*, BCG, *C. albicans*, and SEB (Fig. 4, Datasets S15–S21, and Table S2). The strongest *trans*-eQTL was detected for the *TLR1/6/10* locus, which regulated 105 genes after stimulation with *E. coli*, 80 genes after stimulation with BCG, 7 genes after stimulation with *S. aureus*, and 13 genes after stimulation with SEB. On *E. coli* stimulation, individuals with the TT genotype (rs4833095, T-allele frequency = 0.79) displayed lower expression for many inflammatory response genes (e.g., *IL1B*, *IL6*, *IL12B*) and higher expression for regulatory response genes (e.g., *TGFB1*, *TGFB2*, *IL1RAP*) (Dataset S22). This variant is in strong linkage disequilibrium ($r^2 = 0.89$) in Europeans with an SNP detected as a *trans*-eQTL after the stimulation of monocytes with Pam₃CSK₄ (19).

We also identified a strong *trans*-eQTL located near the *CRI* locus, which regulated 34 genes specifically on *C. albicans* stimulation (Figs. 4 and 6). Individuals with the CC genotype (rs12567990, C-allele frequency = 0.81) (Fig. 6C) had lower levels of expression for 16 genes, including genes involved in the IFN γ -related response (*IFNG*, *STAT1*, *JAK2*, *CXCL10*), and higher expression of 18 genes, including several encoding cell surface proteins (*CLECL5A*, *FCGRT*, *CD14*, *IL-13RA1*) and signaling molecules (e.g., *DUSP4*).

As previous studies have reported that genetic variants can impact gene expression at the steady state in an age- or sex-specific manner (46, 47), we explored how these intrinsic factors affect eQTLs on immune stimulation. We included in the linear mixed model an interaction term, separately for sex (SEX \times SNP) and age (AGE \times SNP), and screened all variants within 1 Mb of each gene. We detected only one significant AGE \times SNP interaction affecting the expression of *SPP1* after stimulation with *E. coli* (rs28628889; $P = 1.3 \times 10^{-8}$, Bonferroni-corrected $P < 0.05$) (Fig. S6.4) and no significant SEX \times SNP interaction. Our analysis had sufficient power to detect loci displaying strong interactions with sex and age but limited power to detect intermediate and weak interactions (Fig. S6.4 and C), suggesting that there are no major differences in the genetic control of immune gene expression between subjects of different ages and sexes.

Collectively, our eQTL analyses revealed that a substantial fraction of the interindividual variation in gene expression can be

attributed to local or *trans*-acting genetic factors, many of which manifest their effects only in the presence of an immunological challenge.

Immune Response eQTLs Are Enriched in Disease Risk Variants. Because a functional role of regulatory variants in disease risk is increasingly recognized (22, 23, 26, 27, 30, 31, 35), we examined the extent to which the detected eQTLs were enriched in genome-wide association study hits (Dataset S23 and Table S3). We found that local and *trans*-eQTLs in the nonstimulated and response states were enriched in disease-associated variants with respect to random sets of SNPs sampled from all variants tested in local eQTL and *trans*-eQTL mapping, respectively (resampling $P < 0.05$). For example, in the absence of stimulation, we confirmed that *NOD2* genetic variation, which has been associated with leprosy (48, 49), affects mRNA levels for this gene (50). We also found that a *TNFSF15-TNFSF8* variant, contributing to inflammatory bowel disease risk (51), strongly affects *TNFSF8* mRNA levels ($P = 3.7 \times 10^{-15}$), while having only a moderate effect on *TNFSF15* ($P = 6.5 \times 10^{-4}$), identifying *TNFSF8* as the probable causal gene.

Remarkably, the observed enrichment in disease-causing variants was stronger for response eQTLs than for eQTLs in the absence of stimulation (4.0–7.3 \times vs. 3.5 \times , respectively) (Table S3). Several response eQTLs at *IRF5*, *ITGAM*, and *IKZF1* have been associated with systemic lupus erythematosus, the pathogenesis of which is linked to an aberrant regulation of innate and adaptive immune response genes (52). Likewise, three loci associated with self-reported allergy and other immune diseases (51, 53) were response eQTLs (Dataset S23), controlling the expression of *SMAD3*, *PTGER4*, and *IKZF3* after stimulation with BCG, *C. albicans*, and IAV, respectively. The strong *trans*-eQTL that we detected at *CRI* (rs12567990) on *C. albicans* stimulation (Fig. 6) has also been associated with interindividual variability in biomarkers of inflammation (erythrocyte sedimentation rate) (54).

Overall, our results indicate that the identification of the genetic factors controlling transcriptional responses to microbial challenges can shed light on the etiology of organismal traits related to infection, inflammation, and autoimmunity.

Quantifying the Impact of Genetic and Nongenetic Factors on Immune Response Variation. Finally, we quantified the respective impacts of age, sex, blood cell heterogeneity, and genetics on transcriptional

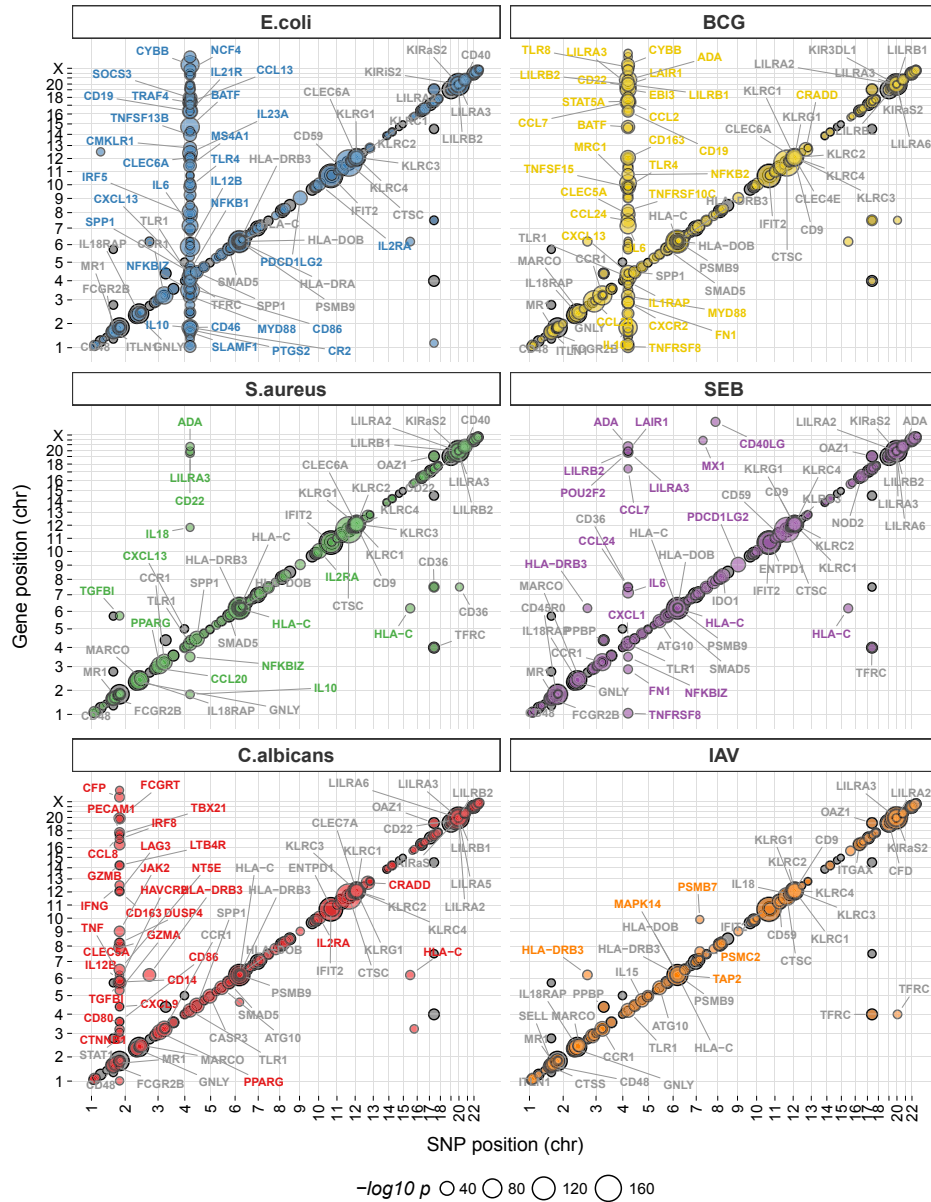


Fig. 4. Local and *trans*-genetic factors associated with gene expression variation. The genomic position of the regulatory SNP is presented on the x axis, whereas that of the gene for which expression variation is associated with the regulatory variant is presented on the y axis. The numbers along the x and y axes are the chromosome numbers. Circles on the diagonals represent genes with expression patterns regulated *in trans*. On each panel, the eQTLs detected in the absence of stimulation were plotted first and were then overlaid with eQTLs detected in the corresponding stimulation conditions. Labels are shown only for genes regulated by local eQTLs with a P value $< 10^{-32}$. For *trans*-eQTLs regulating multiple genes, only the top 30 most significant genes are highlighted. Colored labels indicate genes with significant eQTLs only after stimulation; gray labels indicate the genes with significant eQTLs in both the presence and absence of stimulation. The exact position and rs numbers of both local and *trans*-associated SNPs are provided in [Datasets S8–S21](#).

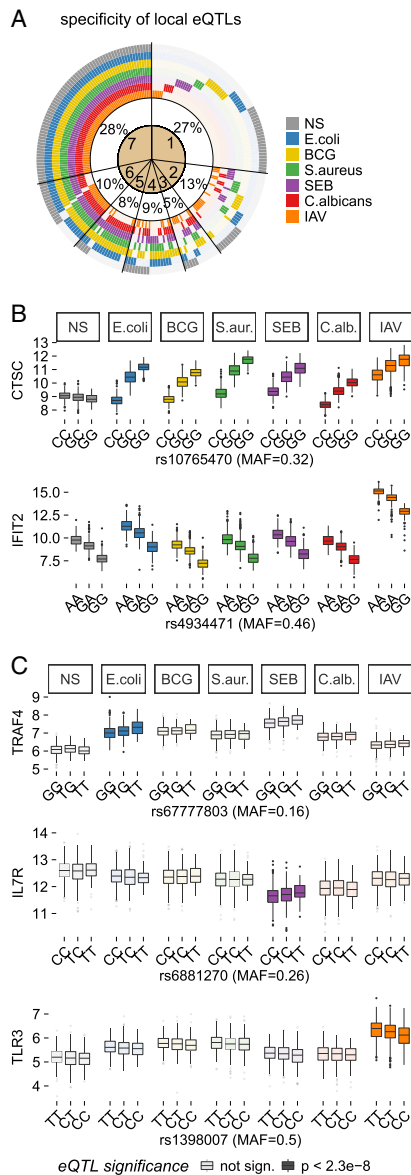


Fig. 5. Stimulus specificity of immune response eQTLs. (A) Specificity of local eQTLs across stimulation conditions. Numbers in the circle sectors (1–7) denote the numbers of stimuli for which the expression of the corresponding genes was associated with a nearby genetic variant. (B) Cases of *CTSC* and *IFIT2* presenting local eQTLs across all seven conditions. The eQTL effect at *CTSC* differed between nonstimulated and stimulated conditions. (C) Cases of *TRAF4*, *IL7R*, and *TLR3* presenting local response eQTLs specific to *E. coli*, SEB, and IAV stimulations, respectively, highlighting G \times E interactions. Gene expression is presented as normalized gene counts. MAF, minor allele frequency; NS, nonstimulated control.

response variation. The largest proportion of gene expression variance was explained by heterogeneity in blood cell composition both in number of genes affected (>400 per condition) and in mean proportion of the variance explained (~18% across genes and conditions) (Fig. 7A and Dataset S24). Age and sex also contributed to the expression variance of a large number of genes (>200 and >300 per condition, respectively), but their individual influences were weak (~2 and ~4%, respectively). Conversely, genetic factors affected the expression of a smaller gene set (~140 per condition for local eQTLs and <40 for *trans*-eQTLs), but their influence was stronger than those of age or sex: ~10 and ~7% for local and *trans*-eQTLs, respectively. The contribution of *trans*-genetic factors was stronger for *E. coli* and BCG than for the other stimuli, affecting ~100 genes and explaining ~10% of their expression variance (Fig. 7A). The genetic component was generally the strongest contributory factor other than heterogeneity in blood cell composition for genes with expression variation associated with genetic factors (Fig. 7B and Fig. S7).

Leaving aside these broad patterns, the respective contributions of nongenetic and genetic factors to immune response variation differed considerably at the individual gene level both within pathways and between stimuli as illustrated by the type 1 IFN and TLR-MyD88 pathways (Fig. 7C). Cellular composition had a stronger impact on expression variation for receptors, such as *IFNAR1/2* and *TLR1/2/4*, than for downstream molecules, with the exception of *Tyk2* and *MyD88*. The impact of genetics also varied considerably between genes: the expression of *IFNAR1* was under the control of a local eQTL, whereas that of *IFNAR2* was regulated by a stimulus-specific *trans*-eQTL. The impact of genetics was particularly strong on *TLR1* expression, where it accounted for up to 21% of the total variance. Age and sex generally had weak effects on gene expression, but several interesting differences were apparent, such as the impact of age on *STAT1* and that of sex on *STAT2* (Fig. 7C).

Together, our analyses indicate that the main source of immune response variation, at least at the transcriptional level, is whole-blood cellular heterogeneity. Age and sex influence the expression of a wide range of genes, but their effect sizes are moderate, whereas genetic factors acting locally or distantly have stronger effects but on a smaller number of immune genes.

Discussion

Using a systems immunology approach in a 1,000-individual healthy cohort specifically designed for the comprehension of the diversity of the human immune system (55), our study represents a systematic investigation of the respective contributions of age, sex, genetics, and cellular heterogeneity to variation in transcriptional responses to immune activation. We found that the variation of immune cell populations in whole blood was the main driver of interindividual differences in immune responses, accounting for ~18% of the total variance in gene expression. The effects of age and sex were overall moderate (<5% of the total variance), consistent with reports based on steady-state expression (24, 25, 56), but they were widespread among immune genes and were generally not mediated by immune cell composition. We also found that age effects were more stimulus-specific compared with those of sex. Although future studies with increased power and the inclusion of donors more than 70 y of age may provide a more nuanced view of their respective effects, our results suggest that the microbial specificity of age effects may be driven by environmental exposures that change throughout life, whereas sex effects are more constant. The detected differences of age effects across stimuli echo recent studies, in which immune cell frequencies were found to be more similar between younger than older monozygotic twins (5) and older individuals presented more heterogeneous immunotypes (i.e., cell populations, cell signaling, and antibody responses) than younger donors in unrelated individuals with ages between 8 and 89 y old (11).

We show that genetic factors affect fewer genes than age or sex but that their effect sizes are stronger (~10% of the total variance) (3, 57). Although the contribution of genetic factors to expression variance can reach even higher values for specific genes or pathways, our findings are in accordance with the moderate influence of genetics in shaping the variation of other immune traits, such as cell proportions (5–7, 15). We nevertheless detected local and *trans*-eQTLs for 43 and 42%, respectively, of the immune genes studied. About 100 genes presented an immune response eQTL, enabling the identification of G × E interactions in the context of infection.

We also identified master regulators of immune responses, including the *trans*-eQTL at *TLRI/6/10*, which we previously detected in monocytes (19). We now extend the description of this *trans*-effect to whole-blood responses to *E. coli*, BCG, and to a lesser extent, *S. aureus* and SEB, highlighting this locus as a major source of immune response variation to bacterial challenges in Europeans. Likewise, the detected *trans*-eQTL at the *CR1* locus reveals a source of variation related to responses to *C. albicans*. CR1 is a receptor for the C3b and C4b split products, opsonins formed as a result of complement system activation. Both *C. albicans* and BCG trigger C3 and C4 cleavage, but only opsonized *C. albicans* engages CR1 (58). We found that *CR1* variation regulated the strength of induced IFN γ responses, downstream signaling pathways (*JAK2/STAT1*), and subsequent chemokine induction (*CXCL10/CXCL9*). The *trans*-eQTL SNP also had a local effect on *CR1* expression (Fig. 6A), but this effect could not account for all of the variance of the *trans*-effect, perhaps reflecting differential temporal regulation as previously observed for the *IFNB1* gene (18). *CR1* variants have been previously associated with differences in erythrocyte sedimentation rate (54, 59), which increases during fungal infections. This highlights the need for studies evaluating the clinical impact of *CR1* variation on susceptibility to fungal infections.

Our age- and sex-stratified cohort allowed us to explore if interactions between these intrinsic variables and the numerous genetic factors identified affect immune responses. With the exception of an AGE × SNP interaction for *E. coli*-induced *SPP1* expression, we found no interactions of age or sex with genetic factors, contrary to recent reports (46, 60). This discrepancy may stem from our focus on immune functions and suggests that the effects of genetic variants on immune gene expression are constant across age groups and between sexes. Consistent with this view, previously reported interactions between age, sex, and genetics did not affect immune genes (with the exception of *NOD2*) (46, 60) or were not identified in whole blood (57).

A nonnegligible fraction of immune response variance remains presently unexplained. Hence, the contribution of other determinants requires additional investigation, including the effects of environmental exposures, epigenetic modifications, interactions with the microbiota, or more complex genetic control (57). In this context, our quantification of the effects of genetic factors on immune response variance (i.e., ~10% on average) should be considered as a conservative estimate, especially if one considers the many regulatory variants with small effect sizes that our analyses cannot detect.

Our study also presents some limitations. Gene expression variation was assessed at 560 immune genes, providing a partial view of the impact that nongenetic and genetic factors have on gene expression *sensu lato*. This choice was based on the robust, highly reproducible gene expression measurements generated by the NanoString arrays in whole blood, avoiding technical variability introduced by amplification steps (38). It was also our strategy to deliberately focus on the expression variation of only immune genes to limit the burden of multiple testing. The use of RNA sequencing will make it possible to

extend these analyses to a wider array of genes and layers of transcriptional variability, such as potential differential isoform usage on infection. Furthermore, analyses of other immune system measurements after stimulation (proteins and metabolites after microbial challenges, antibody responses to vaccines, etc.) are necessary to provide a more comprehensive view of the different intermediate phenotypes that constitute the human immune system.

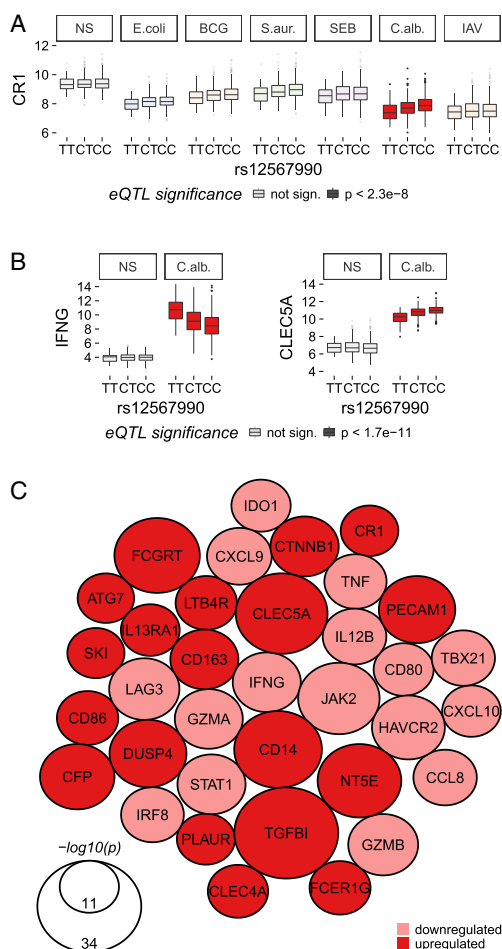


Fig. 6. Stimulus-specific *trans*-acting eQTL at the *CR1* locus. (A) Local eQTL at *CR1* acting specifically in response to *C. albicans* stimulation. (B) rs12567990 was significantly associated, *in trans*, with the expression of *IFNG* and *CLEC5A* only after *C. albicans* stimulation. Gene expression is presented as normalized gene counts. (C) Network of the 34 genes significantly *trans*-regulated by the *CR1* locus ($P < 1.7 \times 10^{-11}$). The size of the nodes is proportional to $-\log_{10}(p)$ of the association between rs12567990 and gene expression. Colors indicate the direction of the change in expression associated with the C allele (frequency = 0.81). NS, non-stimulated control.

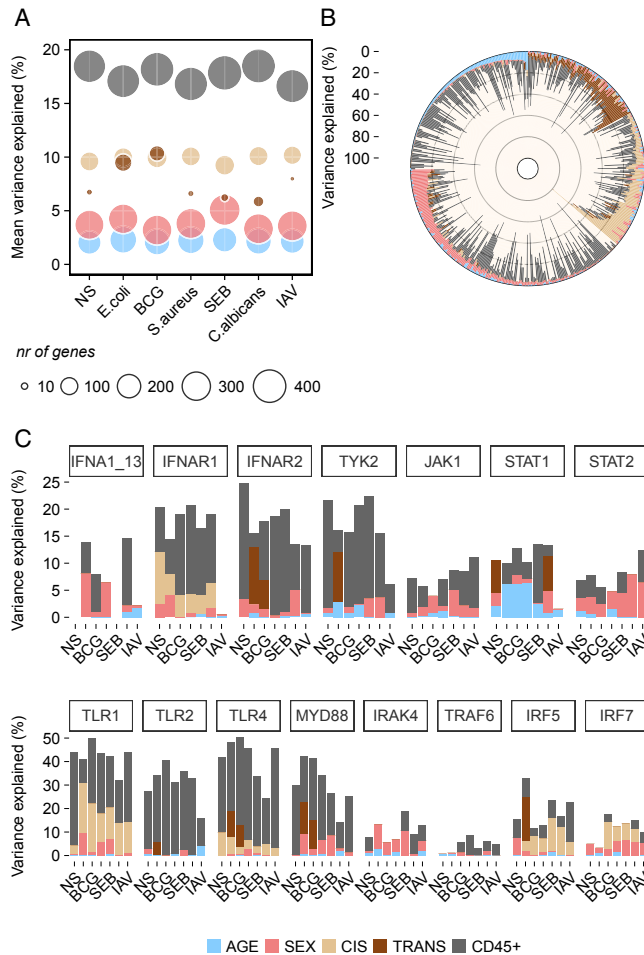


Fig. 7. Expression variance explained by age, sex, genetics, and blood cell composition. (A) Mean variance, across genes, explained by age, sex, genetics, and the proportions of CD45⁺ cell populations in the absence of stimulation and in the six stimulation conditions. The sizes of the circles correspond to the number of genes affected by each factor in each condition. (B) Proportion of the expression variance explained by age, sex, genetics, and proportions of CD45⁺ cells for all genes expressed in response to *E. coli* stimulation. (C) Proportion of the expression variance explained by the different intrinsic and heritable factors for the genes of the type 1 IFN and TLR-MyD88 pathways. The order of stimuli on the x axis is the same as on A. The legend with color-coding applies to A–C. NS, nonstimulated control.

Despite these perceived limitations, the use of gene expression variation and its genetic determinants remains a powerful tool for systems immunology studies, as variants affecting gene expression, in particular after immune stimulation (35), are increasingly recognized as contributing to ultimate organismal phenotypes (22, 26, 27, 30, 31). This notion is strongly supported by the cases of $G \times E$ interactions detected in this study, as response eQTLs exhibited a stronger enrichment in risk variants for human diseases, such as autoimmune and inflammatory disorders, than eQTLs in the nonstimulated state.

Overall, this study provides an assessment of how intrinsic and genetic factors drive interindividual differences in transcriptional responses to bacterial, fungal, and viral challenges. Our dataset is freely accessible via a web-based browser (misage.pasteur.fr/), making it possible to query and visualize the contribution of these factors to immune response variation. It also constitutes a valuable resource for additional exploration in the context of different infection risks or disease outcomes in human populations.

Materials and Methods

The cohort consists of 1,000 healthy volunteers (500 men and 500 women) aged 20–69 y old equally distributed across five decades of life who were selected based on stringent inclusion and exclusion criteria (55). The study was approved by the Comité de Protection des Personnes—Ouest 6 and the Agence Nationale de Sécurité du Médicament and is sponsored by the Institut Pasteur (ID-RCB no. 2012-A00238-35). The study protocol was designed and conducted in accordance with the Declaration of Helsinki and good clinical practice as outlined in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidelines for Good Clinical Practice (https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6/E6_R1_Guideline.pdf), and all subjects gave informed consent. Stimulations were performed on 1 mL whole blood for 22 h using TruCulture tubes (61), and flow cytometry analyses were performed with an eight-color cytometry panel (62). Gene expression was performed using the Human Immunology v2 Gene Expression CodeSet, which contains 594 gene probes that encompass major immune pathways and functions, such as the TLR, Jak-STAT, and MAPK signaling pathways, cytokine–cytokine receptor interactions, apoptosis, and the complement and coagulation cascades.

For each gene in each stimulated condition, a paired *t* test was used to compare expression levels in stimulated and nonstimulated states, controlling for FDR. Seven multilevel regression models were built to estimate the effects of age and sex on gene expression. Structural equation modeling (44) was used to investigate the ways in which the different cell populations mediate the effects of age and sex on gene expression. DNA genotyping was performed using the HumanOmniExpress-24 BeadChip and the HumanExome-12 BeadChip (Illumina). After imputation using the 1,000 Genomes Project imputation reference panel (45), a final dataset of 5,265,361 SNPs was obtained. eQTLs mapping was performed with a linear

mixed model implemented in GenABEL (63). Interaction effects between variables (genetics, sex, and age) on gene expression were estimated using ProbABEL v.0.4.5 (64).

Detailed information about the experimental methods and statistical analyses may be found in *SI Materials and Methods*.

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- Brodin P, Davis MM (2017) Human immune system variation. *Nat Rev Immunol* 17: 21–29.
- De Jager PL, et al. (2015) ImmVar project: Insights and design considerations for future studies of "healthy" immune variation. *Semin Immunol* 27:51–57.
- Liston A, Carr EJ, Linterman MA (2016) Shaping variation in the human immune system. *Trends Immunol* 37:637–646.
- Davis MM, Tatro CM, Furman D (2017) Systems immunology: Just getting started. *Nat Immunol* 18:725–732.
- Brodin P, et al. (2015) Variation in the human immune system is largely driven by non-heritable influences. *Cell* 160:37–47.
- Orrù V, et al. (2013) Genetic variants regulating immune cell levels in health and disease. *Cell* 155:242–256.
- Carr EJ, et al. (2016) The cellular composition of the human immune system is shaped by age and cohabitation. *Nat Immunol* 17:461–468.
- Ter Horst R, et al. (2016) Host and environmental factors influencing individual human cytokine responses. *Cell* 167:1111–1124.e13.
- Shen-Orr SS, et al. (2016) Defective signaling in the JAK-STAT pathway tracks with chronic inflammation and cardiovascular risk in aging humans. *Cell Syst* 3:374–384.e4.
- Furman D, et al. (2014) Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci USA* 111:869–874.
- Kaczorowski KJ, et al. (2017) Continuous immunotypes describe human immune variation and predict diverse responses. *Proc Natl Acad Sci USA* 114:E6097–E6106.
- Blazkova J, et al. (2017) Multicenter systems analysis of human blood reveals immature neutrophils in males and during pregnancy. *J Immunol* 198:2479–2488.
- Li Y, et al. (2016) A functional genomics approach to understand variation in cytokine production in humans. *Cell* 167:1099–1110.e14.
- Li Y, et al. (2016) Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi. *Nat Med* 22:952–960.
- Roederer M, et al. (2015) The genetic architecture of the human immune system: A biosource for autoimmunity and disease pathogenesis. *Cell* 161:387–403.
- Aguirre-Gamboa R, et al. (2016) Differential effects of environmental and genetic factors on T and B cell immune traits. *Cell Rep* 17:2474–2487.
- Lee MN, et al. (2014) Common genetic variants modulate pathogen-sensing responses in human dendritic cells. *Science* 343:1246980.
- Fairfax BP, et al. (2014) Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression. *Science* 343:1246949.
- Quach H, et al. (2016) Genetic adaptation and Neandertal admixture shaped the immune system of human populations. *Cell* 167:643–656.e17.
- Raj T, et al. (2014) Polarization of the effects of autoimmune and neurodegenerative risk alleles in leukocytes. *Science* 344:519–523.
- Nedelec Y, et al. (2016) Genetic ancestry and natural selection drive population differences in immune responses to pathogens. *Cell* 167:657–669.e21.
- Kim-Hellmuth S, et al. (2017) Genetic regulatory effects modified by immune activation contribute to autoimmune disease associations. *Nat Commun* 8:266.
- Fairfax BP, Knight JC (2014) Genetics of gene expression in immunity to infection. *Curr Opin Immunol* 30:63–71.
- Peters MJ, et al., NABECUKBEC Consortium (2015) The transcriptional landscape of age in human peripheral blood. *Nat Commun* 6:8570.
- Jansen R, et al. (2014) Sex differences in the human peripheral blood transcriptome. *BMC Genomics* 15:333.
- Albert FW, Kruglyak L (2015) The role of regulatory variation in complex traits and disease. *Nat Rev Genet* 16:197–212.
- Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M (2012) Linking disease associations with regulatory information in the human genome. *Genome Res* 22: 1748–1759.
- Raj T, et al. (2013) Common risk alleles for inflammatory diseases are targets of recent positive selection. *Am J Hum Genet* 92:517–529.
- Gibson G, Powell JE, Marigorta UM (2015) Expression quantitative trait locus analysis for translational medicine. *Genome Med* 7:60.
- Pickrell JK (2014) Joint analysis of functional genomic data and genome-wide association studies of 18 human traits. *Am J Hum Genet* 94:559–573.
- Li Y, et al. (2016) RNA splicing is a primary link between genetic variation and disease. *Science* 352:600–604.
- Quach H, Quintana-Murci L (2017) Living in an adaptive world: Genomic dissection of the genus *Homo* and its immune response. *J Exp Med* 214:877–894.
- Barreiro LB, et al. (2012) Deciphering the genetic architecture of variation in the immune response to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci USA* 109:1204–1209.
- Çalışkan M, Baker SW, Gilad Y, Ober C (2015) Host genetic variation influences gene expression response to rhinovirus infection. *PLoS Genet* 11:e1005111.
- Alasoo K, et al. (2017) Shared genetic effects on chromatin and gene expression reveal widespread enhancer priming in immune response. *bioRxiv*:10.1101/102392.
- Klein SL, Flanagan KL (2016) Sex differences in immune responses. *Nat Rev Immunol* 16:626–638.
- vom Steeg LG, Klein SL (2016) SexX matters in infectious disease pathogenesis. *PLoS Pathog* 12:e1005374.
- Urrutia A, et al.; Milieu Intérieur Consortium (2016) Standardized whole-blood transcriptional profiling enables the deconvolution of complex induced immune responses. *Cell Rep* 16:2777–2791.
- Viñuela A, Snoek LB, Riksen JA, Kammenga JE (2010) Genome-wide gene expression regulation as a function of genotype and age in *C. elegans*. *Genome Res* 20:929–937.
- Somel M, Khaitovich P, Bahn S, Pääbo S, Lachmann M (2006) Gene expression becomes heterogeneous with age. *Curr Biol* 16:R359–R360.
- Bahar R, et al. (2006) Increased cell-to-cell variation in gene expression in ageing mouse heart. *Nature* 441:1011–1014.
- Sobolev O, et al. (2016) Adjuvanted influenza-H1N1 vaccination reveals lymphoid signatures of age-dependent early responses and of clinical adverse events. *Nat Immunol* 17:204–213.
- Melzer S, et al. (2015) Reference intervals for leukocyte subsets in adults: Results from a population-based study using 10-color flow cytometry. *Cytometry B Clin Cytom* 88: 270–281.
- Gunzler D, Chen T, Wu P, Zhang H (2013) Introduction to mediation analysis with structural equation modeling. *Shanghai Jingshen Yixue* 25:390–394.
- Howie BN, Donnelly P, Marchini J (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet* 5: e1000529.
- Yao C, et al. (2014) Sex- and age-interacting eQTLs in human complex diseases. *Hum Mol Genet* 23:1947–1956.
- Dimas AS, et al.; MuTHER Consortium (2012) Sex-biased genetic effects on gene regulation in humans. *Genome Res* 22:2368–2375.
- Zhang FR, et al. (2009) Genomewide association study of leprosy. *N Engl J Med* 361: 2609–2618.
- Liu H, et al. (2015) Discovery of six new susceptibility loci and analysis of pleiotropic effects in leprosy. *Nat Genet* 47:267–271.
- Naranbhai V, et al. (2015) Genomic modulators of gene expression in human neutrophils. *Nat Commun* 6:7545.
- Liu JZ, et al.; International Multiple Sclerosis Genetics Consortium; International IBD Genetics Consortium (2015) Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* 47:979–986.
- Benthams J, et al. (2015) Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus. *Nat Genet* 47:1457–1464.
- Hinds DA, et al. (2013) A genome-wide association meta-analysis of self-reported allergy identifies shared and allergy-specific susceptibility loci. *Nat Genet* 45:907–911.
- Naitza S, et al. (2012) A genome-wide association scan on the levels of markers of inflammation in Sardinians reveals associations that underpin its complex regulation. *PLoS Genet* 8:e1002480.
- Thomas S, et al.; Milieu Intérieur Consortium (2015) The Milieu Intérieur study—An integrative approach for study of human immunological variance. *Clin Immunol* 157: 277–293.
- Whiting CC, et al. (2015) Large-scale and comprehensive immune profiling and functional analysis of normal human aging. *PLoS One* 10:e0133627.
- Viñuela A, et al. (2016) Age-dependent changes in mean and variance of gene expression across tissues in a twin cohort. *bioRxiv*:10.1101/063883.
- Kozel TR, Brown RR, Pfrommer GS (1987) Activation and binding of C3 by *Candida albicans*. *Infect Immun* 55:1890–1894.
- Kullo IJ, et al. (2011) Complement receptor 1 gene variants are associated with erythrocyte sedimentation rate. *Am J Hum Genet* 89:131–138.
- Kukurba KR, et al. (2016) Impact of the X chromosome and sex on regulatory variation. *Genome Res* 26:768–777.
- Duffy D, et al.; Milieu Intérieur Consortium (2014) Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. *Immunity* 40:436–450.
- Hasan M, et al.; Milieu Intérieur Consortium (2015) Semi-automated and standardized cytometric procedures for multi-panel and multi-parametric whole blood immunophenotyping. *Clin Immunol* 157:261–276.
- Aulchenko YS, Ripke S, Isaacs A, van Duijn CM (2007) GenABEL: An R library for genome-wide association analysis. *Bioinformatics* 23:1294–1296.
- Aulchenko YS, Struchalin MV, van Duijn CM (2010) ProbABEL package for genome-wide association analysis of imputed data. *BMC Bioinformatics* 11:134.

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CLINICAL REPORT



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Novel *DSP* Spectrin 6 Region Variant Causes Neonatal Erythroderma, Failure to Thrive, Severe Herpes Simplex Infections and Brain Lesions

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Desmoplakin (DSP) and Desmoglein 1 (DSG1) variants result in skin barrier defects leading to erythroderma, palmoplantar keratoderma and variable other features. Some DSG1 variant carriers present with SAM syndrome (Severe dermatitis, multiple Allergies, Metabolic wasting) and a SAM-like phenotype has been reported in 4 subjects with different heterozygous DSP variants. We report here a patient with a novel DSP spectrin region (SR) 6 variant c.1756C>T, p.(His586Tyr), novel features of brain lesions and severe recurrent mucocutaneous herpes simplex virus infections, with a favourable response to ustekinumab. Through a review of reported cases of heterozygous variants in DSP SR6 (n = 15) and homozygous or compound heterozygous variants in DSG1 (n = 12) and SAM-like phenotype, we highlight phenotypic variability. Woolly hair, nail abnormalities and cardiomyopathy characterize patients with DSP variants, while elevated immunoglobulin E and food allergies are frequent in patients with DSG1 variants. Clinicians should be aware of the diverse manifestations of desmosomopathies.

Key words: desmoglein; desmoplakin; metabolic wasting; SAM syndrome; severe dermatitis.

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Desmoplakin and desmogleins form the integral parts of desmosomes, which are adhesive intercellular junctions crucial in tissues prone to mechanical stress (e.g. skin, heart, gastrointestinal mucosa) (1). Desmosomal proteins play a role in cell signalling and skin barrier function.

Desmoglein 1 (DSG1) and *Desmoplakin (DSP)* encode critical components of desmosomes, and pathogenic variants in both genes have been implicated in inflammatory skin disorders. *DSG1* variants have been reported recently in patients with designated SAM syndrome

SIGNIFICANCE

Desmoplakin (DSP) and *Desmoglein 1 (DSG1)* gene changes result in skin barrier defects leading to widespread red scaly rash, skin thickening on the palms and soles and variable other features. We report here a patient with a novel *DSP* gene change, novel features of brain lesions and severe viral infections, and a favourable response to treatment with ustekinumab. Woolly hair, nail abnormalities and heart problems characterize patients with *DSP* gene changes, while elevated serum IgE levels and food allergies are frequent in patients with *DSG1* gene changes. Clinicians should be aware of the diverse consequences of *DSP* and *DSG1* gene abnormalities.

(Severe dermatitis, multiple Allergies and Metabolic wasting) with an extremely variable phenotype consisting of severe erythrodermic dermatitis, failure to thrive (FTT), recurrent infections, metabolic wasting, multiple allergies, increased immunoglobulin (Ig) E levels and eosinophilia (2–7).

Two patients with different heterozygous *DSP* variants were reported to have a SAM-like phenotype (8, 9). Autosomal dominant *DSP* variants within the same spectrin 6 (SR6) region also cause erythroderma and cardiomyopathy (10), and palmoplantar keratoderma (PPK) with woolly hair, cardiomyopathy and arrhythmias (11). Recently, 2 additional patients with heterozygous *DSP* SR6 variants have been reported to display SAM syndrome, together with ectodermal dysplasia and cardiomyopathy (coined SAMEC syndrome) (12).

The lack of epithelial barrier proteins commonly seems to lead to immunological dysregulation. Impaired epithelial barrier function enhances Th2 responses and leads to chronic activation of the immune system (13). *DSG1* deficiency results in increased expression of various genes encoding cytokines involved in allergic manifestations, such as *IL5* and *TNF* (2). In SAMEC, *DSG1* was linked to an inability to retain ERBB2-interacting protein (ERBIN) at the cell membrane, probably hampering NF-κB pathway inhibition and promoting epithelial

inflammation (12). Heterozygous *DSP* variants associate with abnormally high levels of proinflammatory cytokines (interleukin (IL) 6, IL-8, and IL-1 β), NF- κ B target gene products, and thymic stromal lymphoprotein in the keratinocytes (12). The dysregulated epidermal immune environment triggers early-onset allergic manifestations beyond the skin, including asthma and allergic rhinoconjunctivitis, as well as eosinophilic oesophagitis and colitis similar to patients with Netherton syndrome (14).

Desmoplakin anchors intermediate filaments to desmosomal plaques and is expressed at high levels in the skin and heart muscle, and at medium levels in bronchi and oesophagus (15). Importantly, individuals with *DSP* and other desmosomal gene variants are at risk of developing cardiomyopathy and lethal arrhythmias, typically at the age of 3–12 years (16), warranting continuous cardiac follow-up.

We report here a patient with a novel *DSP* SR6 region variant (c.1756C>T, p.His586Tyr), presenting with erythrodermic ichthyosis, severe FTT, oesophagitis, recurrent infections, and novel features, such as severe recurrent mucocutaneous herpes simplex virus (HSV) infections, pyogenic granuloma on the tongue, gastritis and brain lesions, further diversifying the *DSP*-related phenotype. We also reviewed all reported patients with heterozygous variants in *DSP* SR6 and with homozygous or compound heterozygous variants in *DSG1* and *SAM/SAMEC*-like phenotype, in order to delineate variable and overlapping clinical features.

METHODS

Written informed consent was obtained from the patient's parents, including permission to publish the photographs.

The immunological assessment, skin histopathology and immunohistochemistry, hair analysis, oesophagus and gastric biopsies, whole-exome sequencing and variant analysis, protein modelling, as well as literature search and statistical analyses, are described in detail in Appendix S1¹.

RESULTS

Case description

Our patient is the first child of non-consanguineous parents of Arab descent, born at term with normal weight and length. At 2 weeks of age, he developed an intertriginous seborrhoeic eczema-like dermatitis (Fig. 1A). A pustular eruption emerged at age 1.5 months and rapidly evolved into a severely pruritic and seemingly painful ichthyosiform erythroderma (Fig. 1B). His hair was normal at birth, but total alopecia developed at 3 months. His nails were abnormally thin and soft.

He had had relapsing *Staphylococcus aureus* skin infections, *Candida albicans* thrush, recurrent enterococcal

and staphylococcal sepsis since the age of 4 months. At 5 months, he developed severe and relapsing protracted HSV-1 stomatitis with extensive mucocutaneous involvement (Fig. 1C). Extensive infectious, immunological and metabolic evaluations were mostly normal and did not suggest any specific immunodeficiency syndrome.

From age 4 months onwards, he developed difficulties with swallowing and progressive FTT. These necessitated percutaneous gastrostomy and initiation of amino acid-based formula feeding. Cow milk protein intolerance was diagnosed at 7 months by an in-hospital oral provocation challenge and manifested as profuse vomiting, diarrhoea and inconsolable crying within hours of administration of standard formula. At 6 months, his serum IgE levels increased up to 958 kU/l (normal <70 kU/l), but then gradually returned to near normal value (102 kU/l). Eosinophil counts remained normal. In the ImmunoCAP specific IgE assay against 112 allergens, only a low-level reactivity (0.7 ISU, normal >0.3 ISU) to prohevein rHev b 6.01 was detected at 9 months, but at 11 months positivity to peanut (0.37 kU/l) and egg-white (1.03 kU/l) appeared. Profound fluid balance disturbances with hypo- and hypernatremia and rapid dehydration or generalized oedema occurred during erythrodermic flares. Arrhythmia or cardiac abnormalities have, so far, not been detected.

Upper gastrointestinal tract endoscopy was normal at 4 months, but revealed lymphocyte and plasmablast mucosal infiltrates in the stomach at 7 months. After several courses of proton pump inhibitors, his gastric histology normalized at 2 years of age. However, he then exhibited mild oesophagitis with some intraepithelial eosinophils, not numerous enough to fulfil the criteria for eosinophilic oesophagitis.

Motor development was delayed from the age of 4 months. Brain magnetic resonance imaging (MRI) at 4 months showed symmetrical diffusion restriction in the superior cerebellar peduncles and decussation. Also, red nuclei in mesencephalon, subthalamic region, globi pallidi and optic radiation were involved (Fig. S1¹). Repeated MRI a month later showed no progression of these changes. His motor development progresses, but remains delayed, with no independent walking at 2 years of age.

At 6 months he developed a firm papular lesion 2.0 cm in diameter on his tongue (Fig. 1D), which resolved spontaneously over months. Biopsy of the lesion revealed a pyogenic granuloma. HSV-1, cytomegalovirus and human papillomavirus (HPV) 11 were found in the lesion by nucleic acid assay, while other viral, bacterial, mycobacterial and fungal investigations were negative.

The patient's skin was treated with basic emollients and courses of topical mild potency corticosteroids. McAleer et al. (8) reported topical tacrolimus, systemic antibiotics, intravenous immunoglobulin (IVIG) therapy and systemic acitretin to be effective in their patient with a *DSP* variant. At 5 months, IVIG therapy for recurrent sepsis was commenced. He has had no bacterial infec-

¹<https://www.medicaljournals.se/acta/content/abstract/10.2340/00015555-3203>



Fig. 1. Clinical features of the reported patient. (A) An intertriginous seborrheic like eczema at 2 weeks of age (B) evolved into an ichthyosiform erythroderma at 1.5 months. (C) Severe and relapsing HSV-1 stomatitis at 5 months and (D) a pyogenic granuloma on the tongue at 6 months. (E) Slight alleviation of scaling and erythema and hair regrowth after initiation of acitretin therapy at 7 months. (F) Ichthyotic erythroderma at 18 months with the development of (G) slight palmoplantar keratoderma (PPK) and (H) onychodystrophy. (I–K) Response to ustekinumab treatment at 25 months included hair regrowth and alleviation of skin scaling and PPK. DSP mutation leads to decreased levels of DSP, DSG1 and KRT10. (L) Skin tissue of a normal control shows prominent DSP antibody staining throughout the epidermis in suprabasal cells, while in the patient (M) only a weak staining is seen in the upper epidermis. DSG1 is distinct intercellularly in (N) healthy skin, but is almost totally lost (O) in the patient. KRT10 stains intensely in (P) the suprabasal healthy skin, but is weak in the patient's upper epidermis and (Q) diminished in the suprabasal layers. Electron microscopy of the patient's skin biopsy samples shows the loss of intercellular cohesiveness, especially (R) suprabasally and decreased numbers of desmosomes and tonofilaments, as well as (S) the absence of perinuclear tonofilaments. Written permission from the parent.

tions since, while recurrent mucocutaneous HSV-1 flares have necessitated continuous acyclovir prophylaxis. IVIG substitution was stopped at 20 months. Acitretin was started at a low dose of 0.25 mg/kg every other day at 7.5 months (8). Acitretin slightly alleviated scaling, erythema and itch and induced hair regrowth (Fig. 1E).

Due to constant vomiting, acitretin was stopped at 14 months, followed by no significant change in his skin condition nor vomiting. His first tooth erupted at 10 months and his teeth have been normal. At 18 months, he developed ichthyotic erythroderma (Fig. 1F–H), and, additionally, he has developed mild PPK and onycho-

dystrophy. His growth has improved substantially due to optimized enteral nutrition.

Since 2 patients with DSP variants were reported to benefit from ustekinumab therapy, with reduction in itching and in dermatitis severity and improvement in hair thickness (9), we initiated ustekinumab treatment at 22 months in our patient. Ustekinumab (0.75 mg/kg) was given initially at 4 weeks and from then on every 12 weeks, leading to alleviation of pruritus, skin scaling and PPK, as well as better hair growth after the first 2 injections (Fig. 1I–K). Oral intake also improved significantly, and gastrostomy use could be discontinued. Along with clinical improvement, serum levels of IL-17A, IL-17F and IL-23 decreased strikingly, whereas concentrations of IL-12p70 somewhat increased, albeit at very low levels (< 1 pg/ml) (Fig. S2¹).

Skin biopsy findings. Skin biopsy from the pustular eruption at 1.5 months showed eczema with a neutrophilic infiltrate and microabscesses. Biopsy at 3 months from the erythrodermic skin revealed a psoriasiform reaction pattern with a dense ichthyosiform stratum corneum and neutrophilic infiltrates in the epidermis and upper dermis. Filaggrin immunostaining was reduced in the first and absent in the second biopsy, suggesting ichthyosis, while LEKTI immunostaining was normal in both biopsies (data not shown). Hair morphology was normal. Immunohistochemistry demonstrated significant decrease in desmoplakin, desmoglein 1 and keratin 10 (Fig. 1L–Q). Skin electron microscopy revealed decreased numbers of desmosomes and tonofilaments, and perinuclear tonofilaments were absent. The loss of intercellular cohesiveness was especially prominent suprabasally (Fig. 1R–S).

Genetic work-up. Ichthyosis and primary immunodeficiency gene panels (Blueprint Genetics, helsinki, Finland) detected no pathogenic variants. Clinical whole-exome sequencing was performed with median sequencing coverage of 132× and 99.1% of target regions were covered with at least 20 reads. Analysis of whole-exome sequence variants revealed a novel heterozygous missense variant in the *DSP* gene: c.1756C>T, p.(His586Tyr) (NM_004415.2), confirmed with bidirectional Sanger sequencing. Based on Sanger sequencing analysis, the parents were not carriers, thus the variant was *de novo* in the patient. This variant has not been reported in the Exome Aggregation Consortium (ExAC) database or Genome Aggregation Database (gnomAD). The variant is predicted deleterious by in silico tools SIFT and MutationTaster and benign by PolyPhen, the CADD score (PHRED-like scaled C-score) is 24.3, suggesting that the amino acid change is deleterious. The His586 residue is located within one of the spectrin repeat domains, SR6, which is a hotspot for dominant missense variants causing cutaneous disease (10). Historical patient with a similar phenotype carried a *de novo* missense variant in the same amino acid residue p.(His586Pro) (8). Protein modelling with the template-based protein structure

tools RaptorX (17) and Swiss-model (18) did not predict alterations in the protein structure. However, histidine is an amino acid with unique chemical properties not particularly well substituted with any other amino acid.

Clinical whole-exome sequencing also identified a variant of uncertain significance, a novel heterozygous in-frame deletion c.11361_11387del, p.(Asp3788_Thr3796del) (NM_001376.4) in *DYNC1H1*, which encodes the heavy chain of cytoplasmic dynein 1 that acts a motor involved in trafficking of vesicles and organelles along microtubule. The variant deletes 9 highly conserved amino acid residues in the motor domain of DYNC1H1 (19). Sanger sequencing of both parents revealed that the variant has occurred *de novo* in the patient.

Literature review on DSP and DSG1 variants and phenotype

The clinical and laboratory characteristics of the patients were compared with heterozygous variants in *DSP* SR6 and with homozygous or compound heterozygous variants in *DSG1* and SAM-like phenotype (Table 1 and Fig. 2). SR6 region dominant *DSP* variants have been reported in 15 patients, with clinical features ranging from a constellation of PPK, woolly hair and arrhythmogenic cardiomyopathy at one end of the spectrum (patients 7–13) to a SAM-like disease with erythroderma, ichthyosis, hypotrichosis, recurrent infections, FTT and gastrointestinal manifestations (patients 1–3, 14) at the other end of the spectrum (Table 1). Overlapping features of cardiomyopathy, erythroderma, ichthyosis, hypotrichosis, PPK and woolly hair have been reported in another 4 patients with *DSP* variants (patients 4–6, 15, Table 1).

Overall, the shared clinical manifestations (*DSP*/*DSG1*) are as follows: PPK (100%/92%), erythroderma (53%/67%), ichthyosis (53%/50%), FTT (40%/33%), recurrent infections (20%/33%), gastrointestinal problems (27%/17%) and developmental delay (20%/25%) (Fig. 2). Otherwise, clinical features varied considerably. Subjects with *DSG1* variants do not manifest eosinophilia, pustulosis, dental and ophthalmic abnormalities, hoarse voice, cardiomyopathy or arrhythmias. Woolly hair and nail abnormalities were more common in patients with *DSP* variants (14/15 vs. 4/12, $p=0.003$ and 11/15 vs. 1/12, $p=0.001$, respectively), while elevated IgE and food allergies were observed more frequently in patients with *DSG1* variants (10/12 vs. 3/15, $p=0.002$ and 8/12 vs. 3/15, $p=0.022$, respectively).

DISCUSSION

We report here a patient with a novel *DSP* variant c.1756C>T, p.His586Tyr and severe erythrodermic ichthyosis, resembling, but distinct from, SAM, with previously undescribed features of severe recurrent

Table 1. Clinical and laboratory characteristics of patients with heterozygous variants in DSP SRG

Pat. No./Ref.	1/ Present case	2/8	3/9	4/10	5/10	6/10	7/11	8/11	9/20	10/20	11/21	12/21	13/22	14/12	15/12
Variant	c.1756 C>T p.H586Y	c.1757 A>C p.H586P	c.1748 T>C p.L583P	c.1846 A>C p.Q616P	c.1852 A>C p.H618P	c.1864 T>C p.L622P	c.1748 T>C p.L583P	c.1691 C>T p.T564I	c.1691 C>T p.Thr564Ile	c.1691 C>T p.Thr564Ile	c.1790 C>T p.Ser597L	c.1790 C>T p.Ser597L	30 bp insertion in codon 608	c.A1757C p.H586P	c.T1828C p.S610P
Exon number	14	14	14	14	14	14	14	13	13	13	14	14	14	14	14
Age (years)	2	9	10	3	8	10	14.5	22	29	10	21	Not reported	18	13	9
Sex	M	M	F	M	M	F	N/A	F	M	M	M	M	F	M	M
Ichthyosis	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+
Erythroderma	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+
PPK	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pruritus	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Pustulosis	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Hypotrichosis	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Woolly hair	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Nail abnormalities	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dental abnormalities	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac abnormalities	-	Aortic stenosis	CMP	LV and RV dilation	LV and RV right atrial dilatation	LV dilation	Aortic stenosis, ventricular CMP	LV and RV dilation	Dilated CMP	Right ventricle infundibulum dilatation	LV dilatation, heart transplant	Dilated CMP	Biventricular CMP	Biventricular dilated CMP	Left-dominant CMP, heart transplant
Arrhythmias	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Recurrent infections	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Failure to thrive	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Gastrointestinal problems	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Developmental delay	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain abnormalities	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
IgE elevation	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Eosinophilia	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
IgE sensitization to allergens	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Food allergies	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Ophthalmic abnormalities	-	-	-	-	large corneal opacities	-	-	-	-	-	-	-	-	-	-
Hoarse voice	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Outcome	Alive	Alive	Alive	Died from heart failure	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Died from heart arrhythmia	Alive	Alive

^aSymmetrical bilateral diffusion modality changes on magnetic resonance imaging, ^blylid dilatation of the lateral and third ventricle, with diffuse prominence of the sulci over both cerebral hemispheres on computed tomography, macrocephaly. CMP: cardiomyopathy; Ig: immunoglobulin; LV: left ventricle; PPK: palmoplantar keratodermis; RV: right ventricle; VSD: ventricular septal defect.

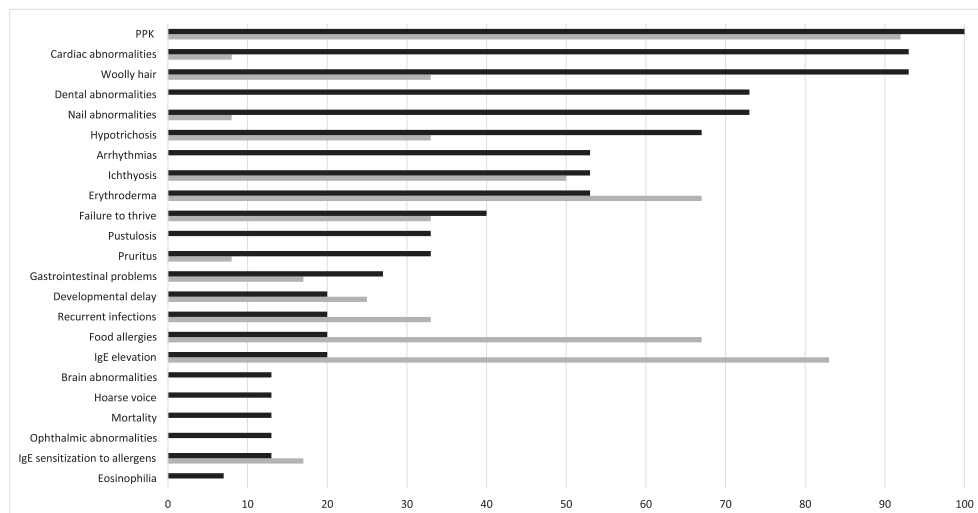


Fig. 2. The prevalence of clinical features in 15 patients with DSP (black bars, Table I) and 12 patients with DSG1 variants (grey bars, references 2–7).

mucocutaneous HSV-1 infection, oral pyogenic granuloma, gastritis and brain lesions. The patient was treated successfully with ustekinumab, targeting the IL-12 and IL-23 as well as downstream IL-17 pathways and adding to the previously reported favourable experience with this medication in another 2 patients with DSP SR6 variants (9). The current patient is thus the third case reported to benefit from ustekinumab therapy.

Patients with SAM present with chronic inflammation of the skin and gastrointestinal epithelium, leading to a dysregulated immune response (9, 12). Our patient showed lymphocytic and plasmablast infiltrates in the gastric mucosa. Coupled with swallowing difficulties and gradually progressing vomiting, as well as further development of mild oesophagitis, these may reflect desmoplakin-related disruption of gastrointestinal mucosa or food intolerance. The severe reaction to cow's milk was out of proportion to his serum IgE levels and resembled food protein-induced enterocolitis syndrome (23).

The aetiology of brain abnormalities in our patient remains unexplained, since DSP is not expressed in the central nervous system (The Human Protein Atlas; GTEx Portal) and since DSP variants have not been associated with brain tissue pathology. Therefore, factors such as undiagnosed perinatal hypoxia, severe FTT and drastic dysnatraemia may contribute. However, developmental delay has been reported in association with some DSP variants (Table I) and its link to desmosomal protein dysfunction warrants further study. Interestingly, our patient had a novel heterozygous *de novo* inframe deletion p.(Asp3788_Thr3796del) in *DYNC1H1*, of which *de novo* variants have been reported in patients with

malformations of cortical development and various other neurological disorders (24, 25). However, the contribution of the *DYNC1H1* variant to aetiology of the observed symmetrical bilateral diffusion modality changes in our patient remains unknown.

The self-resolving tongue pyogenic granuloma in our patient was positive for multiple viral nucleic acids. Although HPV is a well-known cause of mouth papillomatous lesions, both HPV and CMV nucleic acids are frequently detected in oral cavities of healthy infants (26, 27). Instead, HSV-2 and HSV-1 have been reported to cause self-resolving pseudotumour on the tongue (28) and pyogenic granuloma (29), consistent with the preceding HSV-1 mucocutaneous infection in our patient. Also, mechanical stress from a recently introduced pacifier may have contributed to lesion formation (30).

IVIg therapy may have reduced the number of bacterial infections in our patient, as well as in the first described individual with SAM due to DSP variant, despite the normal pretreatment IgG levels in both. Also, IVIG has demonstrated efficacy in patients with Netherton syndrome (31). This may reflect the need for higher IgG levels in patients with epidermal barrier defects who lose protein continuously via inflamed skin. In addition, specific antibody deficiency has been described in some subjects with Netherton syndrome (32). In our patient, IgG levels decreased despite initiation of IVIG, but stabilized after higher and more frequent IVIG dosing. The beneficial effects of IVIG therapy in our case may thus be explained by age-specific immature antibody production exacerbated by chronic protein loss via skin. Acitretin is

successfully used in the management of ichthyoses (33, 34) and was also partially effective in our patient.

Ustekinumab targets the disrupted balance of IL-23 / Th17 pathway and has been efficient in controlling skin inflammation in 2 patients with *DSP* SR6 variants (9), as well as in our case. We provide novel data on the decrease in serum IL-17A and IL-17F levels after initiation of ustekinumab therapy, adding evidence of its biologic efficacy in patients with *DSP* mutations. The increase in IL-12p70 levels, although in levels below 1 pg/ml, during ustekinumab therapy may reflect a compensatory protective effect of IL-12 in skin inflammation (35). Follow-up studies are needed to test the efficacy, if any, of ustekinumab in preventing the development of cardiomyopathy, a fatal complication seen in the majority of *DSP* SR6 variant patients (Table I).

Patients with SR6 *DSP* variants manifest a spectrum of clinical features, ranging from PPK and cardiomyopathy to SAM-like disease. We add severe HSV-1 infection, brain abnormalities and persistent gastrointestinal problems to the diversity of their phenotype. *DSP* variants should be included in the differential diagnosis of infants with ichthyosiform erythroderma, FTT, recurrent infections and these novel features, to allow rapid diagnosis and early therapy. Based on current literature, the clinical features in SAM, SAMEC and SAM-like phenotypes are highly variable. Clinicians caring for these patients should be familiar with the diverse manifestations of desmosomopathies, as well as the importance of early genetic diagnosis, given that only patients with *DSP* SR6, not *DSG1* variants, are at inevitable risk of developing cardiomyopathy. Further studies on the mechanisms of immune dysregulation in patients with *DSG1* and *DSP* variants would provide background for novel targeted therapies, such as immunosuppressants or immunomodulators.

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Conflicts of interest: MM is employed by Blueprint Genetics.

REFERENCES

- McGrath JA. Inherited disorders of desmosomes. *Australas J Dermatol* 2005; 46: 221–229.
- Samuelov L, Sarig O, Harmon RM, Rapaport D, Ishida-Yamamoto A, Isakov O, et al. Desmoglein 1 deficiency results in severe dermatitis, multiple allergies and metabolic wasting. *Nat Genet* 2013; 45: 1244–1248.
- Has C, Jakob T, He Y, Kiritsi D, Hausser I, Bruckner-Tuderman L. Loss of desmoglein 1 associated with palmoplantar keratoderma, dermatitis and multiple allergies. *Br J Dermatol* 2015; 172: 257–261.
- Schlipf NA, Vahlquist A, Teigen N, Virtanen M, Dragomir A, Fismen S, et al. Whole-exome sequencing identifies novel autosomal recessive *DSG1* mutations associated with mild SAM syndrome. *Br J Dermatol* 2016; 174: 444–448.
- Cheng R, Yan M, Ni C, Zhang J, Li M, Yao Z. Report of Chinese family with severe dermatitis, multiple allergies and metabolic wasting syndrome caused by novel homozygous desmoglein-1 gene mutation. *J Dermatol* 2016; 43: 1201–1204.
- Dănescu S, Leppert J, Cosgarea R, Zurac S, Pop S, Baican A, et al. Compound heterozygosity for dominant and recessive *DSG1* mutations in a patient with atypical SAM syndrome (severe dermatitis, multiple allergies, metabolic wasting). *J Eur Acad Dermatol Venereol* 2017; 31: e144–e146.
- Taiber S, Samuelov L, Mohamad J, Cohen Barak E, Sarig O, Shalev SA, et al. SAM syndrome is characterized by extensive phenotypic heterogeneity. *Exp Dermatol* 2018; 27: 787–790.
- McAleer MA, Pohler E, Smith FJ, Wilson NJ, Cole C, MacGowan S, et al. Severe dermatitis, multiple allergies, and metabolic wasting syndrome caused by a novel mutation in the N-terminal plakoin domain of desmoplakin. *J Allergy Clin Immunol* 2015; 136: 1268–1276.
- Paller AS, Czarnowicki T, Renert-Yuval Y, Holland K, Huynh T, Sadlier M, et al. The spectrum of manifestations in desmoplakin gene (*DSP*) spectrin repeat 6 domain mutations: Immunophenotyping and response to ustekinumab. *J Am Acad Dermatol* 2018; 78: 498–505.e2.
- Boyden LM, Kam CY, Hernández-Martin A, Zhou J, Craiglow BG, Sidbury R, et al. Dominant de novo *DSP* mutations cause erythrokeratoderma-cardiomyopathy syndrome. *Hum Mol Genet* 2016; 25: 348–357.
- Keller DI, Stepowski D, Balmer C, Simon F, Guenthard J, Bauer F, et al. De novo heterozygous desmoplakin mutations leading to Naxos-Carvajal disease. *Swiss Med Wkly* 2012; 142: w13670.
- Polivka L, Hadj-Rabia S, Bal E, Leclerc-Mercier S, Madrange M, Hamel Y, et al. Epithelial barrier dysfunction in desmoglein-1 deficiency. *J Allergy Clin Immunol* 2018; 142: 702–706.e7.
- Czarnowicki T, Krueger JG, Guttman-Yassky E. Novel concepts of prevention and treatment of atopic dermatitis through barrier and immune manipulations with implications for the atopic march. *J Allergy Clin Immunol* 2017; 139: 1723–1734.
- Paluel-Marmont C, Bellon N, Barbet P, Leclerc-Mercier S, Hadj-Rabia S, Dupont C, et al. Eosinophilic esophagitis and colonic mucosal eosinophilia in Netherton syndrome. *J Allergy Clin Immunol* 2017; 139: 2003–2005.e1.
- Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. *Science* 2015; 347: 1260419. Human Protein Atlas. Available from: www.proteinatlas.org; <http://www.proteinatlas.org/ENSG00000096696-DSP/tissue>.
- Polivka L, Bodemer C, Hadj-Rabia S. Combination of palmoplantar keratoderma and hair shaft anomalies, the warning signal of severe arrhythmogenic cardiomyopathy: a systematic review on genetic desmosomal diseases. *J Med Genet* 2016; 53: 289–295.
- Källberg M, Wang H, Wang S, Peng J, Wang Z, Lu H, et al. Template-based protein structure modeling using the RaptorX web server. *Nature Protocols* 2012; 7: 1511–1522.
- Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 2014; 42(Web Server issue): W252–258.
- Hoang HT, Schlager MA, Carter AP, Bullock SL. DYNC1H1 mutations associated with neurological diseases compromise processivity of dynein-dynactin-cargo adaptor complexes. *Proc Natl Acad Sci U S A* 2017; 114: E1597–E1606.
- Boulé S, Fressart V, Laux D, Mallet A, Simon F, de Groote P, et al. Expanding the phenotype associated with a desmoplakin dominant mutation: Carvajal/Naxos syndrome associated with leukonychia and oligodontia. *Int J Cardiol* 2012; 161: 50–52.
- Chalabreysse L, Senni F, Bruyère P, Aime B, Ollagnier C, Bozio A, et al. A new hypo/oligodontia syndrome: Carvajal/Naxos

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- syndrome secondary to desmoplakin-dominant mutations. *J Dent Res* 2011; 90: 58–64.
22. Norgett EE, Lucke TW, Bowers B, Munro CS, Leigh IM, Kellsell DP. Early death from cardiomyopathy in a family with autosomal dominant striate palmoplantar keratoderma and woolly hair associated with a novel insertion mutation in desmoplakin. *J Invest Dermatol* 2006; 126: 1651–1654.
 23. Nowak-Węgrzyn A, Chehade M, Groetch ME, Spergel JM, Wood RA, Allen K, et al. International consensus guidelines for the diagnosis and management of food protein-induced enterocolitis syndrome: executive summary-Workgroup Report of the Adverse Reactions to Foods Committee, American Academy of Allergy, Asthma & Immunology. *J Allergy Clin Immunol* 2017; 139: 1111–1126.e4.
 24. Poirier K, Lebrun N, Broix L, Tian G, Saillour Y, Boscheron C, et al. Mutations in TUBG1, DYNC1H1, KIF5C and KIF2A cause malformations of cortical development and microcephaly. *Nat Genet* 2013; 45: 639–647.
 25. Laquerriere A, Maillard C, Cavallin M, Chapon F, Marguet F, Molin A, et al. Neuropathological hallmarks of brain malformations in extreme phenotypes related to DYNC1H1 mutations. *J Neuropathol Exp Neurol* 2017; 76: 195–205.
 26. Martinelli M, Zappa A, Bianchi S, Frati E, Colzani D, Amendola A, et al. Human papillomavirus (HPV) infection and genotype frequency in the oral mucosa of newborns in Milan, Italy. *Clin Microbiol Infect* 2012; 18: E197–199.
 27. Stowell JD, Mask K, Amin M, Clark R, Levis D, Hendley W, et al. Cross-sectional study of cytomegalovirus shedding and immunological markers among seropositive children and their mothers. *BMC Infect Dis* 2014; 14: 568.
 28. Husak R, Tebbe B, Goerd S, Wölfer LU, Zeichardt H, Stöffler-Meilicke M, et al. Pseudotumour of the tongue caused by herpes simplex virus type 2 in an HIV-1 infected immunosuppressed patient. *Br J Dermatol* 1998; 139: 118–121.
 29. El Hayderi L, Paurobally D, Fassotte MF, André J, Arrese JE, Sadzot-Delvaux C, et al. Herpes simplex virus type-I and pyogenic granuloma: a vascular endothelial growth factor-mediated association? *Case Rep Dermatol* 2013; 5: 236–243.
 30. Patil K, Mahima VG, Lahari K. Extralingival pyogenic granuloma. *Indian J Dent Res* 2006; 17: 199–202.
 31. Renner ED, Hartl D, Rylaarsdam S, Young ML, Monaco-Shawver L, Kleiner G, et al. Comèl-Netherton syndrome defined as primary immunodeficiency. *J Allergy Clin Immunol* 2009; 124: 536–543.
 32. Stryk S, Siegfried EC, Knutsen AP. Selective antibody deficiency to bacterial polysaccharide antigens in patients with Netherton syndrome. *Pediatr Dermatol* 1999; 16: 19–22.
 33. Fritsch PO. Retinoids in psoriasis and disorders of keratinization. *J Am Acad Dermatol* 1992; 27: S8–14.
 34. Rajpopat S, Moss C, Mellerio J, Vahlquist A, Ganemo A, Hellstrom-Pigg M, et al. Harlequin ichthyosis: a review of clinical and molecular findings in 45 cases. *Arch Dermatol* 2011; 147: 681–686.
 35. Kulig P, Musiol S, Freiburger SN, Schreiner B, Gyülveszi G, Russo G, et al. IL-12 protects from psoriasisform skin inflammation. *Nat Commun* 2016; 7: 13466.

Part VIII

Bibliography

Bibliography

- [1] R. N. Monastero and S. Pentylala, “Cytokines as biomarkers and their respective clinical cutoff levels,” *International journal of inflammation*, vol. 2017, 2017.
- [2] M. Kopf, M. F. Bachmann, and B. J. Marsland, “Averting inflammation by targeting the cytokine environment,” *Nature Reviews Drug Discovery*, vol. 9, no. 9, pp. 703–718, 2010.
- [3] M. C. Cohen and S. Cohen, “Cytokine function: a study in biologic diversity,” *American journal of clinical pathology*, vol. 105, no. 5, pp. 589–598, 1996.
- [4] Å. Johansson, S. Enroth, M. Palmblad, A. M. Deelder, J. Bergquist, and U. Gyllensten, “Identification of genetic variants influencing the human plasma proteome,” *Proceedings of the National Academy of Sciences*, vol. 110, no. 12, pp. 4673–4678, 2013.
- [5] L. Wu, S. I. Candille, Y. Choi, D. Xie, L. Jiang, J. Li-Pook-Than, H. Tang, and M. Snyder, “Variation and genetic control of protein abundance in humans,” *Nature*, vol. 499, no. 7456, pp. 79–82, 2013.
- [6] S. Enroth, Å. Johansson, S. B. Enroth, and U. Gyllensten, “Strong effects of genetic and lifestyle factors on biomarker variation and use of personalized cutoffs,” *Nature communications*, vol. 5, no. 1, pp. 1–11, 2014.
- [7] P. Brodin, V. Jojic, T. Gao, S. Bhattacharya, C. J. L. Angel, D. Furman, S. Shen-Orr, C. L. Dekker, G. E. Swan, A. J. Butte *et al.*, “Variation in the human immune system is largely driven by non-heritable influences,” *Cell*, vol. 160, no. 1-2, pp. 37–47, 2015.
- [8] Y. Liu, A. Buil, B. C. Collins, L. C. Gillet, L. C. Blum, L.-Y. Cheng, O. Vitek, J. Mouritsen, G. Lachance, T. D. Spector *et al.*, “Quantitative variability of 342 plasma proteins in a human twin population,” *Molecular systems biology*, vol. 11, no. 2, p. 786, 2015.

- [9] W. Zhong, A. Gummesson, A. Tebani, M. J. Karlsson, M.-G. Hong, J. M. Schwenk, F. Edfors, G. Bergström, L. Fagerberg, and M. Uhlén, “Whole-genome sequence association analysis of blood proteins in a longitudinal wellness cohort,” *Genome Medicine*, vol. 12, no. 1, pp. 1–16, 2020.
- [10] L. Folkersen, E. Fauman, M. Sabater-Lleal, R. J. Strawbridge, M. Frånberg, B. Sennblad, D. Baldassarre, F. Veglia, S. E. Humphries, R. Rauramaa *et al.*, “Mapping of 79 loci for 83 plasma protein biomarkers in cardiovascular disease,” *PLoS genetics*, vol. 13, no. 4, p. e1006706, 2017.
- [11] J. Carayol, C. Chabert, A. Di Cara, C. Armenise, G. Lefebvre, D. Langin, N. Viguerie, S. Metairon, W. H. Saris, A. Astrup *et al.*, “Protein quantitative trait locus study in obesity during weight-loss identifies a leptin regulator,” *Nature communications*, vol. 8, no. 1, pp. 1–14, 2017.
- [12] C. Yao, G. Chen, C. Song, J. Keefe, M. Mendelson, T. Huan, B. B. Sun, A. Laser, J. C. Maranville, H. Wu *et al.*, “Genome-wide mapping of plasma protein qtls identifies putatively causal genes and pathways for cardiovascular disease,” *Nature communications*, vol. 9, no. 1, pp. 1–11, 2018.
- [13] R. ter Horst, M. Jaeger, S. P. Smeekens, M. Oosting, M. A. Swertz, Y. Li, V. Kumar, D. A. Diavatopoulos, A. F. Jansen, H. Lemmers *et al.*, “Host and environmental factors influencing individual human cytokine responses,” *Cell*, vol. 167, no. 4, pp. 1111–1124, 2016.
- [14] Y. Li, M. Oosting, S. P. Smeekens, M. Jaeger, R. Aguirre-Gamboa, K. T. Le, P. Deelen, I. Ricaño-Ponce, T. Schoffelen, A. F. Jansen *et al.*, “A functional genomics approach to understand variation in cytokine production in humans,” *Cell*, vol. 167, no. 4, pp. 1099–1110, 2016.
- [15] M. Schirmer, V. Kumar, M. G. Netea, and R. J. Xavier, “The causes and consequences of variation in human cytokine production in health,” *Current opinion in immunology*, vol. 54, pp. 50–58, 2018.
- [16] H.-W. Moon and M. Hur, “Interferon-gamma release assays for the diagnosis of latent tuberculosis infection: an updated review,” *Annals of Clinical & Laboratory Science*, vol. 43, no. 2, pp. 221–229, 2013.

- [17] D. Duffy, V. Rouilly, C. Braudeau, V. Corbière, R. Djebali, M.-N. Ungeheuer, R. Josien, S. T. LaBrie, O. Lantz, D. Louis *et al.*, “Standardized whole blood stimulation improves immunomonitoring of induced immune responses in multi-center study,” *Clinical immunology*, vol. 183, pp. 325–335, 2017.
- [18] C. Bernard, *Leçons sur les phénomènes de la vie commune aux animaux et aux végétaux*. Baillière, 1879, vol. 2.
- [19] S. Thomas, V. Rouilly, E. Patin, C. Alanio, A. Dubois, C. Delval, L.-G. Marquier, N. Fauchoux, S. Sayegrih, M. Vray *et al.*, “The milieu intérieur study—an integrative approach for study of human immunological variance,” *Clinical immunology*, vol. 157, no. 2, pp. 277–293, 2015.
- [20] E. Patin, M. Hasan, J. Bergstedt, V. Rouilly, V. Libri, A. Urrutia, C. Alanio, P. Scepanovic, C. Hammer, F. Jönsson *et al.*, “Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors,” *Nature immunology*, vol. 19, no. 3, pp. 302–314, 2018.
- [21] B. Piasecka, D. Duffy, A. Urrutia, H. Quach, E. Patin, C. Posseme, J. Bergstedt, B. Charbit, V. Rouilly, C. R. MacPherson *et al.*, “Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges,” *Proceedings of the National Academy of Sciences*, vol. 115, no. 3, pp. E488–E497, 2018.
- [22] P. Scepanovic, F. Hodel, S. Mondot, V. Partula, A. Byrd, C. Hammer, C. Alanio, J. Bergstedt, E. Patin, M. Touvier *et al.*, “A comprehensive assessment of demographic, environmental, and host genetic associations with gut microbiome diversity in healthy individuals,” *Microbiome*, vol. 7, no. 1, p. 130, 2019.
- [23] D. Duffy, V. Rouilly, V. Libri, M. Hasan, B. Beitz, M. David, A. Urrutia, A. Bisiaux, S. T. LaBrie, A. Dubois *et al.*, “Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli,” *Immunity*, vol. 40, no. 3, pp. 436–450, 2014.
- [24] A. Urrutia, D. Duffy, V. Rouilly, C. Posseme, R. Djebali, G. Illanes, V. Libri, B. Albaud, D. Gentien, B. Piasecka *et al.*, “Standardized whole-blood transcriptional profiling enables the deconvolution of complex induced immune responses,” *Cell reports*, vol. 16, no. 10, pp. 2777–2791, 2016.

- [25] D. A. Vignali and V. K. Kuchroo, "Il-12 family cytokines: immunological play-makers," *Nature immunology*, vol. 13, no. 8, p. 722, 2012.
- [26] M. Kobayashi, L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri, "Identification and purification of natural killer cell stimulatory factor (nksf), a cytokine with multiple biologic effects on human lymphocytes." *The Journal of experimental medicine*, vol. 170, no. 3, pp. 827–845, 1989.
- [27] A. O. Chua, R. Chizzonite, B. B. Desai, T. P. Truitt, P. Nunes, L. J. Minetti, R. R. Warriar, D. H. Presky, J. F. Levine, and M. K. Gately, "Expression cloning of a human il-12 receptor component. a new member of the cytokine receptor superfamily with strong homology to gp130." *The Journal of Immunology*, vol. 153, no. 1, pp. 128–136, 1994.
- [28] D. H. Presky, H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C.-Y. Wu, M. K. Gately, and U. Gubler, "A functional interleukin 12 receptor complex is composed of two β -type cytokine receptor subunits," *Proceedings of the National Academy of Sciences*, vol. 93, no. 24, pp. 14 002–14 007, 1996.
- [29] S. J. Szabo, S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher, "A novel transcription factor, t-bet, directs th1 lineage commitment," *Cell*, vol. 100, no. 6, pp. 655–669, 2000.
- [30] W. T. Watford, B. D. Hissong, J. H. Bream, Y. Kanno, L. Muul, and J. J. O'Shea, "Signaling by il-12 and il-23 and the immunoregulatory roles of stat4," *Immunological reviews*, vol. 202, no. 1, pp. 139–156, 2004.
- [31] G. Trinchieri, "Interleukin-12 and the regulation of innate resistance and adaptive immunity," *Nature Reviews Immunology*, vol. 3, no. 2, pp. 133–146, 2003.
- [32] D. Sieburth, E. Jabs, J. Warrington, X. Li, J. Lasota, S. LaForgia, K. Kelleher, K. Huebner, J. Wasmuth, and S. Wolf, "Assignment of genes encoding a unique cytokine (il12) composed of two unrelated subunits to chromosomes 3 and 5," *Genomics*, vol. 14, no. 1, pp. 59–62, 1992.
- [33] S. Wolf, P. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, and R. Hewick, "Cloning of cDNA for natural killer cell stimulatory

- factor, a heterodimeric cytokine with multiple biologic effects on t and natural killer cells." *The Journal of Immunology*, vol. 146, no. 9, pp. 3074–3081, 1991.
- [34] R. Jalah, M. Rosati, B. Ganneru, G. R. Pilkington, A. Valentin, V. Kulkarni, C. Bergamaschi, B. Chowdhury, G.-M. Zhang, R. K. Beach *et al.*, "The p40 subunit of interleukin (il)-12 promotes stabilization and export of the p35 subunit implications for improved il-12 cytokine production," *Journal of Biological Chemistry*, vol. 288, no. 9, pp. 6763–6776, 2013.
- [35] G. Carra, F. Gerosa, and G. Trinchieri, "Biosynthesis and posttranslational regulation of human il-12," *The Journal of Immunology*, vol. 164, no. 9, pp. 4752–4761, 2000.
- [36] A. D'Andrea, M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. Chan, M. Kobayashi, D. Young, and E. Nickbarg, "Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells." *The Journal of experimental medicine*, vol. 176, no. 5, pp. 1387–1398, 1992.
- [37] F. Gerosa, B. Baldani-Guerra, L. A. Lyakh, G. Batoni, S. Esin, R. T. Winkler-Pickett, M. R. Consolaro, M. De Marchi, D. Giachino, A. Robbiano *et al.*, "Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells," *The Journal of experimental medicine*, vol. 205, no. 6, pp. 1447–1461, 2008.
- [38] S. E. Macatonia, N. A. Hosken, M. Litton, P. Vieira, C.-S. Hsieh, J. A. Culpepper, M. Wysocka, G. Trinchieri, K. M. Murphy, and A. O'Garra, "Dendritic cells produce il-12 and direct the development of th1 cells from naive cd4+ t cells." *The Journal of Immunology*, vol. 154, no. 10, pp. 5071–5079, 1995.
- [39] M. A. Cassatella, L. Meda, S. Gasperini, A. D'Andrea, X. Ma, and G. Trinchieri, "Interleukin-12 production by human polymorphonuclear leukocytes," *European journal of immunology*, vol. 25, no. 1, pp. 1–5, 1995.
- [40] J. L. Schultze, S. Michalak, J. Lowne, A. Wong, M. H. Gilleece, J. G. Gribben, and L. M. Nadler, "Human non-germinal center b cell interleukin (il)-12 production is primarily regulated by t cell signals cd40 ligand, interferon γ , and il-10: role of b cells in the maintenance of t cell responses," *The Journal of experimental medicine*, vol. 189, no. 1, pp. 1–12, 1999.

- [41] B. Desai, P. Quinn, A. Wolitzky, P. Mongini, R. Chizzonite, and M. Gately, "Il-12 receptor. ii. distribution and regulation of receptor expression." *The Journal of Immunology*, vol. 148, no. 10, pp. 3125–3132, 1992.
- [42] U. Grohmann, M. L. Belladonna, R. Bianchi, C. Orabona, E. Ayroldi, M. C. Fioretti, and P. Puccetti, "Il-12 acts directly on dc to promote nuclear localization of nf- κ b and primes dc for il-12 production," *Immunity*, vol. 9, no. 3, pp. 315–323, 1998.
- [43] L. Rogge, L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler, and F. Sinigaglia, "Selective expression of an interleukin-12 receptor component by human t helper 1 cells," *The Journal of experimental medicine*, vol. 185, no. 5, pp. 825–832, 1997.
- [44] W. E. Thierfelder, J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld *et al.*, "Requirement for stat4 in interleukin-12-mediated responses of natural killer and t cells," *Nature*, vol. 382, no. 6587, pp. 171–174, 1996.
- [45] J. K. Yoo, J. H. Cho, S. W. Lee, and Y. C. Sung, "Il-12 provides proliferation and survival signals to murine cd4+ t cells through phosphatidylinositol 3-kinase/akt signaling pathway," *The Journal of Immunology*, vol. 169, no. 7, pp. 3637–3643, 2002.
- [46] M. W. Teng, E. P. Bowman, J. J. McElwee, M. J. Smyth, J.-L. Casanova, A. M. Cooper, and D. J. Cua, "Il-12 and il-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases," *Nature medicine*, vol. 21, no. 7, p. 719, 2015.
- [47] X. Ma and G. Trinchieri, "Regulation of interleukin-12 production in antigen-presenting cells," *Advances in immunology*, vol. 79, pp. 55–92, 2001.
- [48] Y. Katashiba, R. Miyamoto, A. Hyo, K. Shimamoto, N. Murakami, M. Ogata, R. Amakawa, M. Inaba, S. Nomura, S. Fukuhara *et al.*, "Interferon- α and interleukin-12 are induced, respectively, by double-stranded dna and single-stranded rna in human myeloid dendritic cells," *Immunology*, vol. 132, no. 2, pp. 165–173, 2011.

- [49] M. P. Hayes, F. J. Murphy, and P. R. Burd, "Interferon- γ -dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a tata-containing promoter distinct from the cpg-rich promoter active in epstein-barr virus-transformed lymphoblastoid cells," *Blood, The Journal of the American Society of Hematology*, vol. 91, no. 12, pp. 4645–4651, 1998.
- [50] X. Ma, J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri, "The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells." *The Journal of experimental medicine*, vol. 183, no. 1, pp. 147–157, 1996.
- [51] A. D'Andrea, X. Ma, M. Aste-Amezaga, C. Paganin, and G. Trinchieri, "Stimulatory and inhibitory effects of interleukin (il)-4 and il-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for il-12 and tumor necrosis factor alpha production." *The Journal of experimental medicine*, vol. 181, no. 2, pp. 537–546, 1995.
- [52] Y. Yao, W. Li, M. H. Kaplan, and C.-H. Chang, "Interleukin (il)-4 inhibits il-10 to promote il-12 production by dendritic cells," *The Journal of experimental medicine*, vol. 201, no. 12, pp. 1899–1903, 2005.
- [53] G. Gautier, M. Humbert, F. Deauvieu, M. Scuiller, J. Hiscott, E. E. Bates, G. Trinchieri, C. Caux, and P. Garrone, "A type i interferon autocrine–paracrine loop is involved in toll-like receptor-induced interleukin-12p70 secretion by dendritic cells," *The Journal of experimental medicine*, vol. 201, no. 9, pp. 1435–1446, 2005.
- [54] O. Schulz, A. D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher, and C. R. e Sousa, "Cd40 triggering of heterodimeric il-12 p70 production by dendritic cells in vivo requires a microbial priming signal," *Immunity*, vol. 13, no. 4, pp. 453–462, 2000.
- [55] A. D'andrea, M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri, "Interleukin 10 (il-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/il-12 synthesis in accessory cells." *The Journal of experimental medicine*, vol. 178, no. 3, pp. 1041–1048, 1993.

- [56] C. Du and S. Sriram, "Mechanism of inhibition of lps-induced il-12p40 production by il-10 and $\text{tgf-}\beta$ in ana-1 cells," *Journal of leukocyte biology*, vol. 64, no. 1, pp. 92–97, 1998.
- [57] M. C. Braun and B. L. Kelsall, "Regulation of interleukin-12 production by g-protein-coupled receptors," *Microbes and infection*, vol. 3, no. 2, pp. 99–107, 2001.
- [58] M. Mitsuhashi, J. Liu, S. Cao, X. Shi, and X. Ma, "Regulation of interleukin-12 gene expression and its anti-tumor activities by prostaglandin e2 derived from mammary carcinomas," *Journal of leukocyte biology*, vol. 76, no. 2, pp. 322–332, 2004.
- [59] R. de Jong, F. Altare, I.-A. Haagen, D. G. Elferink, T. de Boer, P. J. van Breda Vriesman, P. J. Kabel, J. M. Draaisma, J. T. van Dissel, F. P. Kroon *et al.*, "Severe mycobacterial and salmonella infections in interleukin-12 receptor-deficient patients," *Science*, vol. 280, no. 5368, pp. 1435–1438, 1998.
- [60] C. Fieschi and J.-L. Casanova, "Mini-review the role of interleukin-12 in human infectious diseases: only a faint signature," *European journal of immunology*, vol. 33, no. 6, pp. 1461–1464, 2003.
- [61] M. Dalod, T. P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Brière, G. Trinchieri, and C. A. Biron, "Interferon α/β and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo," *The Journal of experimental medicine*, vol. 195, no. 4, pp. 517–528, 2002.
- [62] T. Hamza, J. B. Barnett, and B. Li, "Interleukin 12 a key immunoregulatory cytokine in infection applications," *International journal of molecular sciences*, vol. 11, no. 3, pp. 789–806, 2010.
- [63] P. Ling, M. K. Gately, U. Gubler, A. S. Stern, P. Lin, K. Hollfelder, C. Su, Y. Pan, and J. Hakimi, "Human il-12 p40 homodimer binds to the il-12 receptor but does not mediate biologic activity." *The Journal of Immunology*, vol. 154, no. 1, pp. 116–127, 1995.
- [64] F. Mattner, S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rüde, and T. Germann, "The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer," *European journal of immunology*, vol. 23, no. 9, pp. 2202–2208, 1993.

- [65] S. J. Ha, C. H. Lee, S. B. Lee, C. M. Kim, K. L. Jang, H. S. Shin, and Y. C. Sung, "A novel function of il-12p40 as a chemotactic molecule for macrophages," *The Journal of Immunology*, vol. 163, no. 5, pp. 2902–2908, 1999.
- [66] S. A. Khader, S. Partida-Sanchez, G. Bell, D. M. Jelley-Gibbs, S. Swain, J. E. Pearl, N. Ghilardi, F. J. Desauvage, F. E. Lund, and A. M. Cooper, "Interleukin 12p40 is required for dendritic cell migration and t cell priming after mycobacterium tuberculosis infection," *The Journal of experimental medicine*, vol. 203, no. 7, pp. 1805–1815, 2006.
- [67] T. Germann, E. Rüde, F. Mattner, and M. Gately, "The il-12 p40 homodimer as a specific antagonist of the il-12 heterodimer." *Immunology today*, vol. 16, no. 10, pp. 500–501, 1995.
- [68] F. P. Heinzl, A. M. Hujer, F. N. Ahmed, and R. M. Rerko, "In vivo production and function of il-12 p40 homodimers." *The Journal of Immunology*, vol. 158, no. 9, pp. 4381–4388, 1997.
- [69] M. Jana, S. Dasgupta, R. N. Saha, X. Liu, and K. Pahan, "Induction of tumor necrosis factor- α (tnf- α) by interleukin-12 p40 monomer and homodimer in microglia and macrophages," *Journal of neurochemistry*, vol. 86, no. 2, pp. 519–528, 2003.
- [70] K. Pahan, F. G. Sheikh, X. Liu, S. Hilger, M. McKinney, and T. M. Petro, "Induction of nitric-oxide synthase and activation of nf- κ b by interleukin-12 p40 in microglial cells," *Journal of Biological Chemistry*, vol. 276, no. 11, pp. 7899–7905, 2001.
- [71] S. Brahmachari and K. Pahan, "Suppression of regulatory t cells by il-12p40 homodimer via nitric oxide," *The Journal of Immunology*, vol. 183, no. 3, pp. 2045–2058, 2009.
- [72] T. D. Russell, Q. Yan, G. Fan, A. P. Khalifah, D. K. Bishop, S. L. Brody, and M. J. Walter, "Il-12 p40 homodimer-dependent macrophage chemotaxis and respiratory viral inflammation are mediated through il-12 receptor β_1 ," *The Journal of Immunology*, vol. 171, no. 12, pp. 6866–6874, 2003.

- [73] C. L. Mikols, L. Yan, J. Y. Norris, T. D. Russell, A. P. Khalifah, R. R. Hachem, M. M. Chakinala, R. D. Yusen, M. Castro, E. Kuo *et al.*, "Il-12 p80 is an innate epithelial cell effector that mediates chronic allograft dysfunction," *American journal of respiratory and critical care medicine*, vol. 174, no. 4, pp. 461–470, 2006.
- [74] J. R. Piccotti, S. Y. Chan, K. Li, E. J. Eichwald, and D. K. Bishop, "Differential effects of il-12 receptor blockade with il-12 p40 homodimer on the induction of cd4+ and cd8+ ifn-gamma-producing cells." *The Journal of Immunology*, vol. 158, no. 2, pp. 643–648, 1997.
- [75] A. P. Nigg, S. Zahn, D. Ruckerl, C. Hölscher, T. Yoshimoto, J. M. Ehrchen, F. Wölbing, M. C. Udey, and E. Von Stebut, "Dendritic cell-derived il-12p40 homodimer contributes to susceptibility in cutaneous leishmaniasis in balb/c mice," *The Journal of Immunology*, vol. 178, no. 11, pp. 7251–7258, 2007.
- [76] S.-Y. Lee, Y. O. Jung, D.-J. Kim, C.-M. Kang, Y.-M. Moon, Y.-J. Heo, H.-J. Oh, S.-J. Park, S.-H. Yang, S. K. Kwok *et al.*, "Il-12p40 homodimer ameliorates experimental autoimmune arthritis," *The Journal of Immunology*, vol. 195, no. 7, pp. 3001–3010, 2015.
- [77] D.-J. Kim, K.-S. Kim, M.-Y. Song, S.-H. Seo, S.-J. Kim, B.-G. Yang, M.-H. Jang, and Y.-C. Sung, "Delivery of il-12p40 ameliorates dss-induced colitis by suppressing il-17a expression and inflammation in the intestinal mucosa," *Clinical immunology*, vol. 144, no. 3, pp. 190–199, 2012.
- [78] B. Oppmann, R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh *et al.*, "Novel p19 protein engages il-12p40 to form a cytokine, il-23, with biological activities similar as well as distinct from il-12," *Immunity*, vol. 13, no. 5, pp. 715–725, 2000.
- [79] C. Parham, M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K. P. Singh, F. Vega *et al.*, "A receptor for the heterodimeric cytokine il-23 is composed of il-12 β 1 and a novel cytokine receptor subunit, il-23 γ ," *The Journal of Immunology*, vol. 168, no. 11, pp. 5699–5708, 2002.
- [80] N. J. Wilson, K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel *et al.*, "Development, cytokine

- profile and function of human interleukin 17-producing helper t cells,” *Nature immunology*, vol. 8, no. 9, pp. 950–957, 2007.
- [81] S. C. Liang, X.-Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser, “Interleukin (il)-22 and il-17 are coexpressed by th17 cells and cooperatively enhance expression of antimicrobial peptides,” *The Journal of experimental medicine*, vol. 203, no. 10, pp. 2271–2279, 2006.
- [82] S. L. Gaffen, R. Jain, A. V. Garg, and D. J. Cua, “The il-23–il-17 immune axis: from mechanisms to therapeutic testing,” *Nature reviews immunology*, vol. 14, no. 9, pp. 585–600, 2014.
- [83] C. Tang, S. Chen, H. Qian, and W. Huang, “Interleukin-23: as a drug target for autoimmune inflammatory diseases,” *Immunology*, vol. 135, no. 2, pp. 112–124, 2012.
- [84] Y. Li, H. Wang, H. Lu, and S. Hua, “Regulation of memory t cells by interleukin-23,” *International archives of allergy and immunology*, vol. 169, no. 3, pp. 157–162, 2016.
- [85] J. Chang, T. J. Voorhees, Y. Liu, Y. Zhao, and C.-H. Chang, “Interleukin-23 production in dendritic cells is negatively regulated by protein phosphatase 2a,” *Proceedings of the National Academy of Sciences*, vol. 107, no. 18, pp. 8340–8345, 2010.
- [86] R. E. Roses, S. Xu, M. Xu, U. Koldovsky, G. Koski, and B. J. Czerniecki, “Differential production of il-23 and il-12 by myeloid-derived dendritic cells in response to tlr agonists,” *The Journal of Immunology*, vol. 181, no. 7, pp. 5120–5127, 2008.
- [87] S. Louis, C.-A. Dutertre, L. Vimeux, L. Fery, L. Henno, S. Diocou, S. Kahi, C. Deveau, L. Meyer, C. Goujard *et al.*, “Il-23 and il-12p70 production by monocytes and dendritic cells in primary hiv-1 infection,” *Journal of leukocyte biology*, vol. 87, no. 4, pp. 645–653, 2010.
- [88] J. Jin, X. Xie, Y. Xiao, H. Hu, Q. Zou, X. Cheng, and S.-C. Sun, “Epigenetic regulation of the expression of il12 and il23 and autoimmune inflammation by the deubiquitinase trabd,” *Nature immunology*, vol. 17, no. 3, p. 259, 2016.
- [89] E. Lee, W. L. Trepicchio, J. L. Oestreicher, D. Pittman, F. Wang, F. Chamian, M. Dhodapkar, and J. G. Krueger, “Increased expression of interleukin 23 p19 and

- p40 in lesional skin of patients with psoriasis vulgaris," *The Journal of experimental medicine*, vol. 199, no. 1, pp. 125–130, 2004.
- [90] E. J. Yang, M. P. Smith, K. Ly, and T. Bhutani, "Evaluating guselkumab: an anti-il-23 antibody for the treatment of plaque psoriasis," *Drug design, development and therapy*, vol. 13, p. 1993, 2019.
- [91] T. Nagai, O. Devergne, T. F. Mueller, D. L. Perkins, J. M. van Seventer, and G. A. van Seventer, "Timing of ifn- β exposure during human dendritic cell maturation and naive th cell stimulation has contrasting effects on th1 subset generation: a role for ifn- β -mediated regulation of il-12 family cytokines and il-18 in naive th cell differentiation," *The Journal of Immunology*, vol. 171, no. 10, pp. 5233–5243, 2003.
- [92] A. Isaacs, J. Lindenmann, and R. C. Valentine, "Virus interference. ii. some properties of interferon," *Proceedings of the Royal Society of London. Series B-Biological Sciences*, vol. 147, no. 927, pp. 268–273, 1957.
- [93] M. Rubinstein, S. Rubinstein, P. C. Familletti, M. S. Gross, R. S. Miller, A. A. Waldman, and S. Pestka, "Human leukocyte interferon purified to homogeneity," *Science*, vol. 202, no. 4374, pp. 1289–1290, 1978.
- [94] C. M. L. de Padilla and T. B. Niewold, "The type i interferons: Basic concepts and clinical relevance in immune-mediated inflammatory diseases," *Gene*, vol. 576, no. 1, pp. 14–21, 2016.
- [95] S. Pestka, "The interferons: 50 years after their discovery, there is much more to learn," *Journal of Biological Chemistry*, vol. 282, no. 28, pp. 20 047–20 051, 2007.
- [96] M. Miyamoto, T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi, "Regulated expression of a gene encoding a nuclear factor, irf-1, that specifically binds to ifn- β gene regulatory elements," *Cell*, vol. 54, no. 6, pp. 903–913, 1988.
- [97] H. Harada, T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi, "Structurally similar but functionally distinct factors, irf-1 and irf-2, bind to the same regulatory elements of ifn and ifn-inducible genes," *Cell*, vol. 58, no. 4, pp. 729–739, 1989.

- [98] S. Scheu, P. Dresing, and R. M. Locksley, "Visualization of $\text{ifn}\beta$ production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo," *Proceedings of the National Academy of Sciences*, vol. 105, no. 51, pp. 20 416–20 421, 2008.
- [99] P. Li, J. J.-Y. Wong, C. Sum, W.-X. Sin, K.-Q. Ng, M. B. Koh, and K.-C. Chin, "Irf8 and irf3 cooperatively regulate rapid interferon- β induction in human blood monocytes," *Blood, The Journal of the American Society of Hematology*, vol. 117, no. 10, pp. 2847–2854, 2011.
- [100] F. P. Siegal, N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y.-J. Liu, "The nature of the principal type 1 interferon-producing cells in human blood," *Science*, vol. 284, no. 5421, pp. 1835–1837, 1999.
- [101] Y.-J. Liu, "Ipc: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors," *Annu. Rev. Immunol.*, vol. 23, pp. 275–306, 2005.
- [102] C. Richez, K. Yasuda, A. A. Watkins, S. Akira, R. Lafyatis, J. M. van Seventer, and I. R. Rifkin, "Tlr4 ligands induce $\text{ifn}\alpha$ production by mouse conventional dendritic cells and human monocytes after $\text{ifn}\beta$ priming," *The Journal of Immunology*, vol. 182, no. 2, pp. 820–828, 2009.
- [103] E. Jaks, M. Gavutis, G. Uzé, J. Martal, and J. Piehler, "Differential receptor subunit affinities of type 1 interferons govern differential signal activation," *Journal of molecular biology*, vol. 366, no. 2, pp. 525–539, 2007.
- [104] L. Velazquez, M. Fellous, G. R. Stark, and S. Pellegrini, "A protein tyrosine kinase in the interferon $\alpha\beta$ signaling pathway," *Cell*, vol. 70, no. 2, pp. 313–322, 1992.
- [105] M. Müller, J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler *et al.*, "The protein tyrosine kinase jak1 complements defects in interferon- α/β and- γ signal transduction," *Nature*, vol. 366, no. 6451, pp. 129–135, 1993.
- [106] G. R. Stark and J. E. Darnell Jr, "The jak-stat pathway at twenty," *Immunity*, vol. 36, no. 4, pp. 503–514, 2012.
- [107] D. E. Levy, D. S. Kessler, R. Pine, N. Reich, and J. Darnell, "Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and

- negative transcriptional control." *Genes & development*, vol. 2, no. 4, pp. 383–393, 1988.
- [108] X.-Y. Fu, D. S. Kessler, S. A. Veals, D. E. Levy, and J. Darnell, "Isgf3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains." *Proceedings of the National Academy of Sciences*, vol. 87, no. 21, pp. 8555–8559, 1990.
- [109] C. Schindler, X.-Y. Fu, T. Improta, R. Aebersold, and J. E. Darnell, "Proteins of transcription factor isgf-3: one gene encodes the 91- and 84-kda isgf-3 proteins that are activated by interferon alpha." *Proceedings of the National Academy of Sciences*, vol. 89, no. 16, pp. 7836–7839, 1992.
- [110] I. Rusinova, S. Forster, S. Yu, A. Kannan, M. Masse, H. Cumming, R. Chapman, and P. J. Hertzog, "Interferome v2. 0: an updated database of annotated interferon-regulated genes," *Nucleic acids research*, vol. 41, no. D1, pp. D1040–D1046, 2012.
- [111] J. W. Schoggins, S. J. Wilson, M. Panis, M. Y. Murphy, C. T. Jones, P. Bieniasz, and C. M. Rice, "A diverse range of gene products are effectors of the type i interferon antiviral response," *Nature*, vol. 472, no. 7344, pp. 481–485, 2011.
- [112] L. C. Platanius and E. N. Fish, "Signaling pathways activated by interferons," *Experimental hematology*, vol. 27, no. 11, pp. 1583–1592, 1999.
- [113] G. M. Boxx and G. Cheng, "The roles of type i interferon in bacterial infection," *Cell host & microbe*, vol. 19, no. 6, pp. 760–769, 2016.
- [114] L. Icardi, R. Mori, V. Gesellchen, S. Eyckerman, L. De Cauwer, J. Verhelst, K. Vercauteren, X. Saelens, P. Meuleman, G. Leroux-Roels *et al.*, "The sin3a repressor complex is a master regulator of stat transcriptional activity," *Proceedings of the National Academy of Sciences*, vol. 109, no. 30, pp. 12 058–12 063, 2012.
- [115] L. B. Ivashkiv and L. T. Donlin, "Regulation of type i interferon responses," *Nature reviews Immunology*, vol. 14, no. 1, pp. 36–49, 2014.
- [116] S. Uddin, B. Majchrzak, J. Woodson, P. Arunkumar, Y. Alsayed, R. Pine, P. R. Young, E. N. Fish, and L. C. Platanius, "Activation of the p38 mitogen-activated

- protein kinase by type i interferons,” *Journal of Biological Chemistry*, vol. 274, no. 42, pp. 30 127–30 131, 1999.
- [117] Y. Li, S. Batra, A. Sassano, B. Majchrzak, D. E. Levy, M. Gaestel, E. N. Fish, R. J. Davis, and L. C. Platanias, “Activation of mitogen-activated protein kinase kinase (mkk) 3 and mkk6 by type i interferons,” *Journal of Biological Chemistry*, vol. 280, no. 11, pp. 10 001–10 010, 2005.
- [118] I. A. Mayer, A. Verma, I. M. Grumbach, S. Uddin, F. Lekmine, F. Ravandi, B. Majchrzak, S. Fujita, E. N. Fish, and L. C. Platanias, “The p38 mapk pathway mediates the growth inhibitory effects of interferon- α in bcr-abl-expressing cells,” *Journal of Biological Chemistry*, vol. 276, no. 30, pp. 28 570–28 577, 2001.
- [119] H. Ishida, K. Ohkawa, A. Hosui, N. Hiramatsu, T. Kanto, K. Ueda, T. Takehara, and N. Hayashi, “Involvement of p38 signaling pathway in interferon- α -mediated antiviral activity toward hepatitis c virus,” *Biochemical and biophysical research communications*, vol. 321, no. 3, pp. 722–727, 2004.
- [120] L. C. Platanias, “Mechanisms of type-i-and type-ii-interferon-mediated signalling,” *Nature Reviews Immunology*, vol. 5, no. 5, pp. 375–386, 2005.
- [121] N. A. De Weerd, J. P. Vivian, T. K. Nguyen, N. E. Mangan, J. A. Gould, S.-J. Braniff, L. Zaker-Tabrizi, K. Y. Fung, S. C. Forster, T. Beddoe *et al.*, “Structural basis of a unique interferon- β signaling axis mediated via the receptor ifnar1,” *Nature immunology*, vol. 14, no. 9, p. 901, 2013.
- [122] N. A. de Weerd, A. Y. Matthews, P. R. Pattie, N. M. Bourke, S. S. Lim, J. P. Vivian, J. Rossjohn, and P. J. Hertzog, “A hot spot on interferon α/β receptor subunit 1 (ifnar1) underpins its interaction with interferon- β and dictates signaling,” *Journal of Biological Chemistry*, vol. 292, no. 18, pp. 7554–7565, 2017.
- [123] K.-I. Arimoto, S. Miyauchi, S. A. Stoner, J.-B. Fan, and D.-E. Zhang, “Negative regulation of type i ifn signaling,” *Journal of Leukocyte Biology*, vol. 103, no. 6, pp. 1099–1116, 2018.
- [124] M. Sato, N. Hata, M. Asagiri, T. Nakaya, T. Taniguchi, and N. Tanaka, “Positive feedback regulation of type i ifn genes by the ifn-inducible transcription factor irf-7,” *FEBS letters*, vol. 441, no. 1, pp. 106–110, 1998.

- [125] F. Ma, B. Li, Y. Yu, S. S. Iyer, M. Sun, and G. Cheng, "Positive feedback regulation of type i interferon by the interferon-stimulated gene sting," *EMBO reports*, vol. 16, no. 2, pp. 202–212, 2015.
- [126] K. Senger, M. Merika, T. Agaloti, J. Yie, C. R. Escalante, G. Chen, A. K. Aggarwal, and D. Thanos, "Gene repression by coactivator repulsion," *Molecular cell*, vol. 6, no. 4, pp. 931–937, 2000.
- [127] J. R. Teijaro, "Type i interferons in viral control and immune regulation," *Current opinion in virology*, vol. 16, pp. 31–40, 2016.
- [128] M. Rotger, K. K. Dang, J. Fellay, E. L. Heinzen, S. Feng, P. Descombes, K. V. Shianna, D. Ge, H. F. Günthard, D. B. Goldstein *et al.*, "Genome-wide mrna expression correlates of viral control in cd4+ t-cells from hiv-1-infected individuals," *PLoS pathogens*, vol. 6, no. 2, 2010.
- [129] A. Murira and A. Lamarre, "Type-i interferon responses: from friend to foe in the battle against chronic viral infection," *Frontiers in immunology*, vol. 7, p. 609, 2016.
- [130] G. Trinchieri, "Type i interferon: friend or foe?" *Journal of Experimental Medicine*, vol. 207, no. 10, pp. 2053–2063, 2010.
- [131] M. Biggioggero, L. Gabbriellini, and P. L. Meroni, "Type i interferon therapy and its role in autoimmunity," *Autoimmunity*, vol. 43, no. 3, pp. 248–254, 2010.
- [132] Y. Ioannou and D. A. Isenberg, "Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy," *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, vol. 43, no. 7, pp. 1431–1442, 2000.
- [133] D. Chaussabel, C. Quinn, J. Shen, P. Patel, C. Glaser, N. Baldwin, D. Stichweh, D. Blankenship, L. Li, I. Munagala *et al.*, "A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus," *Immunity*, vol. 29, no. 1, pp. 150–164, 2008.
- [134] M. K. Crow and J. Wohlgemuth, "Microarray analysis of gene expression in lupus," *Arthritis Res Ther*, vol. 5, no. 6, p. 279, 2003.

- [135] G. I. Rice, I. Melki, M.-L. Frémond, T. A. Briggs, M. P. Rodero, N. Kitabayashi, A. Oojageer, B. Bader-Meunier, A. Belot, C. Bodemer *et al.*, “Assessment of type I interferon signaling in pediatric inflammatory disease,” *Journal of clinical immunology*, vol. 37, no. 2, pp. 123–132, 2017.
- [136] E. F. Remmers, R. M. Plenge, A. T. Lee, R. R. Graham, G. Hom, T. W. Behrens, P. I. De Bakker, J. M. Le, H.-S. Lee, F. Batliwalla *et al.*, “Stat4 and the risk of rheumatoid arthritis and systemic lupus erythematosus,” *New England Journal of Medicine*, vol. 357, no. 10, pp. 977–986, 2007.
- [137] J. Banchereau and V. Pascual, “Type I interferon in systemic lupus erythematosus and other autoimmune diseases,” *Immunity*, vol. 25, no. 3, pp. 383–392, 2006.
- [138] J. C. Hall and A. Rosen, “Type I interferons: crucial participants in disease amplification in autoimmunity,” *Nature Reviews Rheumatology*, vol. 6, no. 1, p. 40, 2010.
- [139] D. Choubey and K. D. Moudgil, “Interferons in autoimmune and inflammatory diseases: regulation and roles,” *Journal of Interferon & Cytokine Research*, vol. 31, no. 12, pp. 857–865, 2011.
- [140] C. T. Ng, B. M. Sullivan, J. R. Teijaro, A. M. Lee, M. Welch, S. Rice, K. C. Sheehan, R. D. Schreiber, and M. B. Oldstone, “Blockade of interferon beta, but not interferon alpha, signaling controls persistent viral infection,” *Cell host & microbe*, vol. 17, no. 5, pp. 653–661, 2015.
- [141] D. J. Gough, N. L. Messina, C. J. Clarke, R. W. Johnstone, and D. E. Levy, “Constitutive type I interferon modulates homeostatic balance through tonic signaling,” *Immunity*, vol. 36, no. 2, pp. 166–174, 2012.
- [142] K. E. Balashov, D. R. Smith, S. J. Khoury, D. A. Hafler, and H. L. Weiner, “Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand,” *Proceedings of the National Academy of Sciences*, vol. 94, no. 2, pp. 599–603, 1997.
- [143] R. Mastrangeli, F. D’Amici, C.-W. D’Acunzio, S. Fiumi, M. Rossi, M. Terlizze, W. Palinsky, and H. Bierau, “A deamidated interferon- β variant binds to integrin $\alpha v \beta 3$,” *Cytokine*, vol. 104, pp. 38–41, 2018.

- [144] Ú. A. Rossi, L. M. Finocchiaro, and G. C. Glikin, "Interferon- β gene transfer inhibits melanoma cells adhesion and migration," *Cytokine*, vol. 89, pp. 201–208, 2017.
- [145] M. R. Doherty, H. Cheon, D. J. Junk, S. Vinayak, V. Varadan, M. L. Telli, J. M. Ford, G. R. Stark, and M. W. Jackson, "Interferon-beta represses cancer stem cell properties in triple-negative breast cancer," *Proceedings of the National Academy of Sciences*, vol. 114, no. 52, pp. 13 792–13 797, 2017.
- [146] E. Kalie, D. A. Jaitin, Y. Podoplelova, J. Piehler, and G. Schreiber, "The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities," *Journal of Biological Chemistry*, vol. 283, no. 47, pp. 32 925–32 936, 2008.
- [147] S. Strain, S. W. Fesik, and I. M. Armitage, "Characterization of lipopolysaccharide from a heptoseless mutant of escherichia coli by carbon 13 nuclear magnetic resonance." *Journal of Biological Chemistry*, vol. 258, no. 5, pp. 2906–2910, 1983.
- [148] T. Vatanen, A. D. Kostic, E. d’Hennezel, H. Siljander, E. A. Franzosa, M. Yassour, R. Kolde, H. Vlamakis, T. D. Arthur, A.-M. Hämäläinen *et al.*, "Variation in microbiome lps immunogenicity contributes to autoimmunity in humans," *Cell*, vol. 165, no. 4, pp. 842–853, 2016.
- [149] H. Salden and B. M. Bas, "Endotoxin binding to platelets in blood from patients with a sepsis syndrome," *Clinical chemistry*, vol. 40, no. 8, pp. 1575–1579, 1994.
- [150] C. J. Kelly, S. P. Colgan, and D. N. Frank, "Of microbes and meals: the health consequences of dietary endotoxemia," *Nutrition in Clinical Practice*, vol. 27, no. 2, pp. 215–225, 2012.
- [151] T. M. Wassenaar and K. Zimmermann, "Lipopolysaccharides in food, food supplements, and probiotics: should we be worried?" *European Journal of Microbiology and Immunology*, vol. 8, no. 3, pp. 63–69, 2018.
- [152] K. de Punder and L. Pruimboom, "Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability," *Frontiers in immunology*, vol. 6, p. 223, 2015.

- [153] J. N. Fullerton, E. Segre, R. P. De Maeyer, A. A. Maini, and D. W. Gilroy, "Intravenous endotoxin challenge in healthy humans: an experimental platform to investigate and modulate systemic inflammation," *JoVE (Journal of Visualized Experiments)*, no. 111, p. e53913, 2016.
- [154] C. Sauter and C. Wolfensberger, "Interferon in human serum after injection of endotoxin." *Lancet (London, England)*, vol. 2, no. 8199, pp. 852–853, 1980.
- [155] A. Zariri and P. van der Ley, "Biosynthetically engineered lipopolysaccharide as vaccine adjuvant," *Expert review of vaccines*, vol. 14, no. 6, pp. 861–876, 2015.
- [156] J. J. Seeley and S. Ghosh, "Molecular mechanisms of innate memory and tolerance to lps," *Journal of leukocyte biology*, vol. 101, no. 1, pp. 107–119, 2017.
- [157] A. Poltorak, X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos *et al.*, "Defective lps signaling in c3h/vej and c57bl/10scsr mice: mutations in tlr4 gene," *Science*, vol. 282, no. 5396, pp. 2085–2088, 1998.
- [158] M. Muzio, D. Bosisio, N. Polentarutti, G. D'amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena *et al.*, "Differential expression and regulation of toll-like receptors (tlr) in human leukocytes: selective expression of tlr3 in dendritic cells," *The Journal of Immunology*, vol. 164, no. 11, pp. 5998–6004, 2000.
- [159] A. Visintin, A. Mazzoni, J. H. Spitzer, D. H. Wyllie, S. K. Dower, and D. M. Segal, "Regulation of toll-like receptors in human monocytes and dendritic cells," *The Journal of Immunology*, vol. 166, no. 1, pp. 249–255, 2001.
- [160] V. Hornung, S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdörfer, T. Giese, S. Endres, and G. Hartmann, "Quantitative expression of toll-like receptor 1–10 mrna in cellular subsets of human peripheral blood mononuclear cells and sensitivity to cpg oligodeoxynucleotides," *The Journal of Immunology*, vol. 168, no. 9, pp. 4531–4537, 2002.
- [161] K. J. Kieser and J. C. Kagan, "Multi-receptor detection of individual bacterial products by the innate immune system," *Nature Reviews Immunology*, vol. 17, no. 6, p. 376, 2017.

- [162] J. C. Kagan, T. Su, T. Horng, A. Chow, S. Akira, and R. Medzhitov, "Tram couples endocytosis of toll-like receptor 4 to the induction of interferon- β ," *Nature immunology*, vol. 9, no. 4, pp. 361–368, 2008.
- [163] Y.-C. Lu, W.-C. Yeh, and P. S. Ohashi, "Lps/tlr4 signal transduction pathway," *Cytokine*, vol. 42, no. 2, pp. 145–151, 2008.
- [164] E. M. Pålsson-McDermott and L. A. O'Neill, "Signal transduction by the lipopolysaccharide receptor, toll-like receptor-4," *Immunology*, vol. 113, no. 2, pp. 153–162, 2004.
- [165] Y. Li, S.-L. Deng, Z.-X. Lian, and K. Yu, "Roles of toll-like receptors in nitroxidative stress in mammals," *Cells*, vol. 8, no. 6, p. 576, 2019.
- [166] Z. Jiang, P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos *et al.*, "Cdi4 is required for myd88-independent lps signaling," *Nature immunology*, vol. 6, no. 6, pp. 565–570, 2005.
- [167] I. Zanoni, R. Ostuni, L. R. Marek, S. Barresi, R. Barbalat, G. M. Barton, F. Granucci, and J. C. Kagan, "Cdi4 controls the lps-induced endocytosis of toll-like receptor 4," *Cell*, vol. 147, no. 4, pp. 868–880, 2011.
- [168] I. Zanoni, R. Ostuni, G. Capuano, M. Collini, M. Caccia, A. E. Ronchi, M. Rocchetti, F. Mingozzi, M. Foti, G. Chirico *et al.*, "Cdi4 regulates the dendritic cell life cycle after lps exposure through nfat activation," *Nature*, vol. 460, no. 7252, pp. 264–268, 2009.
- [169] S. Dunzendorfer, H.-K. Lee, K. Soldau, and P. S. Tobias, "Tlr4 is the signaling but not the lipopolysaccharide uptake receptor," *The Journal of Immunology*, vol. 173, no. 2, pp. 1166–1170, 2004.
- [170] I. Zanoni and F. Granucci, "Differences in lipopolysaccharide-induced signaling between conventional dendritic cells and macrophages," *Immunobiology*, vol. 215, no. 9-10, pp. 709–712, 2010.
- [171] S. Das, K. A. Owen, K. T. Ly, D. Park, S. G. Black, J. M. Wilson, C. D. Sifri, K. S. Ravichandran, P. B. Ernst, and J. E. Casanova, "Brain angiogenesis inhibitor 1 (bair) is a pattern recognition receptor that mediates macrophage binding and

- engulfment of gram-negative bacteria,” *Proceedings of the National Academy of Sciences*, vol. 108, no. 5, pp. 2136–2141, 2011.
- [172] E. A. Billings, C. S. Lee, K. A. Owen, R. S. D’Souza, K. S. Ravichandran, and J. E. Casanova, “The adhesion gpcr bait mediates macrophage ros production and microbicidal activity against gram-negative bacteria,” *Science signaling*, vol. 9, no. 413, pp. ra14–ra14, 2016.
- [173] B.-H. Kim, J. D. Chee, C. J. Bradfield, E.-S. Park, P. Kumar, and J. D. MacMicking, “Interferon-induced guanylate-binding proteins in inflammasome activation and host defense,” *Nature immunology*, vol. 17, no. 5, pp. 481–489, 2016.
- [174] J. A. Hagar, D. A. Powell, Y. Aachoui, R. K. Ernst, and E. A. Miao, “Cytoplasmic lps activates caspase-11: implications in tlr4-independent endotoxic shock,” *Science*, vol. 341, no. 6151, pp. 1250–1253, 2013.
- [175] N. Kayagaki, M. T. Wong, I. B. Stowe, S. R. Ramani, L. C. Gonzalez, S. Akashi-Takamura, K. Miyake, J. Zhang, W. P. Lee, A. Muszyński *et al.*, “Noncanonical inflammasome activation by intracellular lps independent of tlr4,” *Science*, vol. 341, no. 6151, pp. 1246–1249, 2013.
- [176] J. Shi, Y. Zhao, K. Wang, X. Shi, Y. Wang, H. Huang, Y. Zhuang, T. Cai, F. Wang, and F. Shao, “Cleavage of gsdmd by inflammatory caspases determines pyroptotic cell death,” *Nature*, vol. 526, no. 7575, pp. 660–665, 2015.
- [177] S. Sriskandan and D. Altmann, “The immunology of sepsis,” *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland*, vol. 214, no. 2, pp. 211–223, 2008.
- [178] R. S. Hotchkiss and I. E. Karl, “The pathophysiology and treatment of sepsis,” *New England Journal of Medicine*, vol. 348, no. 2, pp. 138–150, 2003.
- [179] G. S. Ling, J. Bennett, K. Woollard, M. Szajna, L. Fossati-Jimack, P. Taylor, D. Scott, G. Franzoso, H. Cook, and M. Botto, “Integrin cd11b positively regulates tlr4-induced signalling pathways in dendritic cells but not in macrophages,” *Nature communications*, vol. 5, p. 3039, 01 2014.

- [180] J.-M. Cavillon, C. Adrie, C. Fitting, and M. Adib-Conquy, "Reprogramming of circulatory cells in sepsis and sirs," *Journal of endotoxin research*, vol. 11, no. 5, pp. 311–320, 2005.
- [181] A. O'Garra, P. S. Redford, F. W. McNab, C. I. Bloom, R. J. Wilkinson, and M. P. Berry, "The immune response in tuberculosis," *Annual review of immunology*, vol. 31, pp. 475–527, 2013.
- [182] W. H. Organization *et al.*, "Global tb report," World Health Organization, Tech. Rep., 2019.
- [183] M. Pai, M. Behr, D. Dowdy, K. Dheda, M. Divangahi, C. Boehme, and M. Ravignione, "Tuberculosis. nature reviews disease primers, 2, 16076," 2016.
- [184] P. K. Drain, K. L. Bajema, D. Dowdy, K. Dheda, K. Naidoo, S. G. Schumacher, S. Ma, E. Meermeier, D. M. Lewinsohn, and D. R. Sherman, "Incipient and sub-clinical tuberculosis: a clinical review of early stages and progression of infection," *Clinical microbiology reviews*, vol. 31, no. 4, 2018.
- [185] J. R. Starke, "Tuberculin skin test versus the interferon- γ release assays: Out with the old, in with the new," *Pediatrics*, vol. 145, no. 1, 2020.
- [186] D. Duffy, E. Nemes, A. Llibre, V. Rouilly, E. Filander, H. Africa, S. Mabwe, L. Jaxa, B. Charbit, M. Musvosvi *et al.*, "Immune profiling in m. tuberculosis infection enables stratification of patients with active disease," *bioRxiv*, p. 581298, 2019.
- [187] J. Keane, S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun, "Tuberculosis associated with infliximab, a tumor necrosis factor α -neutralizing agent," *New England Journal of Medicine*, vol. 345, no. 15, pp. 1098–1104, 2001.
- [188] H. Yamada, S. Mizumo, R. Horai, Y. Iwakura, and I. Sugawara, "Protective role of interleukin-1 in mycobacterial infection in il-1 α/β double-knockout mice," *Laboratory investigation*, vol. 80, no. 5, pp. 759–767, 2000.
- [189] J. L. Flynn, J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom, "An essential role for interferon gamma in resistance to mycobacterium tuberculo-

- sis infection." *The Journal of experimental medicine*, vol. 178, no. 6, pp. 2249–2254, 1993.
- [190] L. Moreira-Teixeira, K. Mayer-Barber, A. Sher, and A. O'Garra, "Type I interferons in tuberculosis: Foe and occasionally friend," *Journal of Experimental Medicine*, vol. 215, no. 5, pp. 1273–1285, 2018.
- [191] M. P. Berry, C. M. Graham, F. W. McNab, Z. Xu, S. A. Bloch, T. Oni, K. A. Wilkinson, R. Banchereau, J. Skinner, R. J. Wilkinson *et al.*, "An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis," *Nature*, vol. 466, no. 7309, pp. 973–977, 2010.
- [192] A. Singhania, R. Verma, C. M. Graham, J. Lee, T. Tran, M. Richardson, P. Lecine, P. Leissner, M. P. Berry, R. J. Wilkinson *et al.*, "A modular transcriptional signature identifies phenotypic heterogeneity of human tuberculosis infection," *Nature communications*, vol. 9, no. 1, pp. 1–17, 2018.
- [193] G. Zhang, N. A. Deweerdt, S. A. Stifter, L. Liu, B. Zhou, W. Wang, Y. Zhou, B. Ying, X. Hu, A. Y. Matthews *et al.*, "A proline deletion in *ifn γ* impairs *ifn*-signaling and underlies increased resistance to tuberculosis in humans," *Nature communications*, vol. 9, no. 1, pp. 1–9, 2018.
- [194] C. Manca, L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J. M. Musser, C. E. Barry, V. H. Freedman, and G. Kaplan, "Virulence of a mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce *th1* type immunity and is associated with induction of *ifn*- α/β ," *Proceedings of the National Academy of Sciences*, vol. 98, no. 10, pp. 5752–5757, 2001.
- [195] D. Ordway, M. Henao-Tamayo, M. Harton, G. Palanisamy, J. Troudt, C. Shanley, R. J. Basaraba, and I. M. Orme, "The hypervirulent mycobacterium tuberculosis strain Hn878 induces a potent *th1* response followed by rapid down-regulation," *The Journal of Immunology*, vol. 179, no. 1, pp. 522–531, 2007.
- [196] S. Giosue, M. Casarini, L. Alemanno, G. Galluccio, P. Mattia, G. Pedicelli, L. Rebeck, A. Bisetti, and F. Ameglio, "Effects of aerosolized interferon- α in patients with pulmonary tuberculosis," *American journal of respiratory and critical care medicine*, vol. 158, no. 4, pp. 1156–1162, 1998.

- [197] P. Zarogoulidis, I. Kioumis, N. Papanas, K. Manika, T. Kontakiotis, A. Papagianis, and K. Zarogoulidis, "The effect of combination ifn-alpha-2a with usual antituberculosis chemotherapy in non-responding tuberculosis and diabetes mellitus: a case report and review of the literature," *Journal of Chemotherapy*, vol. 24, no. 3, pp. 173–177, 2012.
- [198] N. Ruangkiattikul, A. Nerlich, K. Abdissa, S. Lienenklaus, A. Suwandi, N. Janze, K. Laarmann, J. Spanier, U. Kalinke, S. Weiss *et al.*, "cgas-sting-tbki-irf3/7 induced interferon- β contributes to the clearing of non tuberculous mycobacterial infection in mice," *Virulence*, vol. 8, no. 7, pp. 1303–1315, 2017.
- [199] J. Kuchtey, S. A. Fulton, S. M. Reba, C. V. Harding, and W. H. Boom, "Interferon- $\alpha\beta$ mediates partial control of early pulmonary mycobacterium bovis bacillus calmette–guérin infection," *Immunology*, vol. 118, no. 1, pp. 39–49, 2006.
- [200] C. M. Ward, H. Jyonouchi, S. V. Kotenko, S. V. Smirnov, R. Patel, H. Aguila, G. McSherry, B. Dashefsky, and S. M. Holland, "Adjunctive treatment of disseminated mycobacterium avium complex infection with interferon alpha-2b in a patient with complete interferon-gamma receptor $\gamma 1$ deficiency," *European journal of pediatrics*, vol. 166, no. 9, pp. 981–985, 2007.
- [201] L. Desvignes, A. J. Wolf, and J. D. Ernst, "Dynamic roles of type i and type ii ifns in early infection with mycobacterium tuberculosis," *The Journal of Immunology*, vol. 188, no. 12, pp. 6205–6215, 2012.
- [202] F. W. McNab, J. Ewbank, A. Howes, L. Moreira-Teixeira, A. Martirosyan, N. Ghilardi, M. Saraiva, and A. O'Garra, "Type i ifn induces il-10 production in an il-27-independent manner and blocks responsiveness to ifn- γ for production of il-12 and bacterial killing in mycobacterium tuberculosis-infected macrophages," *The Journal of Immunology*, vol. 193, no. 7, pp. 3600–3612, 2014.
- [203] M. L. Donovan, T. E. Schultz, T. J. Duke, and A. Blumenthal, "Type i interferons in the pathogenesis of tuberculosis: molecular drivers and immunological consequences," *Frontiers in immunology*, vol. 8, p. 1633, 2017.
- [204] S. Zaltron, A. Spinetti, L. Biasi, C. Baiguera, and F. Castelli, "Chronic hcv infection: epidemiological and clinical relevance," *BMC infectious diseases*, vol. 12, no. 2, pp. 1–7, 2012.

- [205] C. W. Spearman and M. W. Sonderup, "Direct-acting antiviral therapy in sub-Saharan Africa." *The Lancet. Gastroenterology & Hepatology*, vol. 4, no. 2, p. 85, 2019.
- [206] S. Pol, "Virus de l'hépatite C-25 ans, la fin de l'histoire?" *médecine/sciences*, vol. 29, no. 11, pp. 998–1003, 2013.
- [207] W. H. Organization *et al.*, *Global hepatitis report 2017*. World Health Organization, 2017.
- [208] E. D. Bethea, Q. Chen, C. Hur, R. T. Chung, and J. Chhatwal, "Should we treat acute hepatitis C? a decision and cost-effectiveness analysis," *Hepatology*, vol. 67, no. 3, pp. 837–846, 2018.
- [209] N. Scott, D. P. Wilson, A. J. Thompson, E. Barnes, M. El-Sayed, A. S. Benzaken, H. E. Drummer, and M. E. Hellard, "The case for a universal hepatitis C vaccine to achieve hepatitis C elimination," *BMC medicine*, vol. 17, no. 1, p. 175, 2019.
- [210] E. Thomas, V. D. Gonzalez, Q. Li, A. A. Modi, W. Chen, M. Nouredin, Y. Rotman, and T. J. Liang, "HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons," *Gastroenterology*, vol. 142, no. 4, pp. 978–988, 2012.
- [211] H.-H. Hoffmann, W. M. Schneider, and C. M. Rice, "Interferons and viruses: an evolutionary arms race of molecular interactions," *Trends in immunology*, vol. 36, no. 3, pp. 124–138, 2015.
- [212] R. Firdaus, A. Biswas, K. Saha, A. Mukherjee, F. Pal, S. Chaudhuri, A. Chandra, A. Konar, and P. C. Sadhukhan, "Modulation of TLR 3, 7 and 8 expressions in HCV genotype 3 infected individuals: potential correlations of pathogenesis and spontaneous clearance," *BioMed research international*, vol. 2014, 2014.
- [213] K. Machida, K. T. Cheng, V. M.-H. Sung, A. M. Levine, S. Fong, and M. M. Lai, "Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6," *Journal of virology*, vol. 80, no. 2, pp. 866–874, 2006.
- [214] S. Zhang, B. Saha, K. Kodys, and G. Szabo, "IFN- γ production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells," *Journal of hepatology*, vol. 59, no. 3, pp. 442–449, 2013.

- [215] D. Moonka, K. A. Milkovich, B. Rodriguez, M. Abouljoud, M. M. Lederman, and D. D. Anthony, "Hepatitis c virus-specific t-cell gamma interferon and proliferative responses are more common in perihepatic lymph nodes than in peripheral blood or liver," *Journal of virology*, vol. 82, no. 23, pp. 11742–11748, 2008.
- [216] G. Missale, R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M. G. Rumi, M. Houghton, F. Fiaccadori, C. Ferrari *et al.*, "Different clinical behaviors of acute hepatitis c virus infection are associated with different vigor of the anti-viral cell-mediated immune response." *The Journal of clinical investigation*, vol. 98, no. 3, pp. 706–714, 1996.
- [217] H. M. Diepolder, R. Zachoval, R. M. Hoffmann, E. A. Wierenga, T. Santantonio, M.-C. Jung, D. Eichenlaub, and G. R. Pape, "Possible mechanism involving t-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis c virus infection." *Lancet (London, England)*, vol. 346, no. 8981, p. 1006, 1995.
- [218] S. Smyk-Pearson, I. A. Tester, J. Klarquist, B. E. Palmer, J.-M. Pawlotsky, L. Golden-Mason, and H. R. Rosen, "Spontaneous recovery in acute human hepatitis c virus infection: functional t-cell thresholds and relative importance of cd4 help," *Journal of virology*, vol. 82, no. 4, pp. 1827–1837, 2008.
- [219] A. I. Su, J. P. Pezacki, L. Wodicka, A. D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz *et al.*, "Genomic analysis of the host response to hepatitis c virus infection," *Proceedings of the National Academy of Sciences*, vol. 99, no. 24, pp. 15669–15674, 2002.
- [220] A. García-Sastre, "Ten strategies of interferon evasion by viruses," *Cell host & microbe*, vol. 22, no. 2, pp. 176–184, 2017.
- [221] E. Meylan, J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp, "Cardif is an adaptor protein in the rig-i antiviral pathway and is targeted by hepatitis c virus," *Nature*, vol. 437, no. 7062, pp. 1167–1172, 2005.
- [222] K. Li, E. Foy, J. C. Ferreon, M. Nakamura, A. C. Ferreon, M. Ikeda, S. C. Ray, M. Gale, and S. M. Lemon, "Immune evasion by hepatitis c virus ns3/4a protease-mediated cleavage of the toll-like receptor 3 adaptor protein trif," *Proceedings of the National Academy of Sciences*, vol. 102, no. 8, pp. 2992–2997, 2005.

- [223] J. M. Pestka, M. B. Zeisel, E. Bläser, P. Schürmann, B. Bartosch, F.-L. Cosset, A. H. Patel, H. Meisel, J. Baumert, S. Viazov *et al.*, “Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis c,” *Proceedings of the National Academy of Sciences*, vol. 104, no. 14, pp. 6025–6030, 2007.
- [224] J. Nattermann, G. Feldmann, G. Ahlenstiel, B. Langhans, T. Sauerbruch, and U. Spengler, “Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis c,” *Gut*, vol. 55, no. 6, pp. 869–877, 2006.
- [225] M. H. Heim and R. Thimme, “Innate and adaptive immune responses in hcv infections,” *Journal of hepatology*, vol. 61, no. 1, pp. S14–S25, 2014.
- [226] S.-H. Park and B. Rehermann, “Immune responses to hcv and other hepatitis viruses,” *Immunity*, vol. 40, no. 1, pp. 13–24, 2014.
- [227] H. M. Aldesouki, H. M. Zidan, and E. M. Elashry, “Serum il-12p70 and tlr 7 gene expression in egyptian patients infected with hcv and treated with sofosbuvir, ribavirin and/or pegylated interferon.”
- [228] S. Yoneda, T. Umemura, Y. Katsuyama, A. Kamijo, S. Joshita, M. Komatsu, T. Ichijo, A. Matsumoto, K. Yoshizawa, M. Ota *et al.*, “Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis c patients,” *Journal of infectious diseases*, vol. 203, no. 8, pp. 1087–1095, 2011.
- [229] J. A. Quiroga, J. Martín, S. Navas, and V. Carreno, “Induction of interleukin-12 production in chronic hepatitis c virus infection correlates with the hepatocellular damage,” *Journal of Infectious Diseases*, vol. 178, no. 1, pp. 247–251, 1998.
- [230] A. Alhethel, A. Albarrag, Z. Shakoor, K. Alswat, A. Abdo, and W. Al-Hamoudi, “Assessment of pro-inflammatory cytokines in sera of patients with hepatitis c virus infection before and after anti-viral therapy,” *The Journal of Infection in Developing Countries*, vol. 10, no. 10, pp. 1093–1098, 2016.
- [231] M. P. Hayes, J. Wang, and M. A. Norcross, “Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes,” *Blood*, 1995.

- [232] K. Abdi, N. Singh, and P. Matzinger, "T-cell control of il-12p75 production," *Scandinavian journal of immunology*, vol. 64, no. 2, pp. 83–92, 2006.
- [233] A. Snijders, P. Kalinski, C. Hilkens, and M. L. Kapsenberg, "High-level il-12 production by human dendritic cells requires two signals." *International immunology*, vol. 10, no. 11, pp. 1593–1598, 1998.
- [234] J. Ren, X. Chen, and Z. J. Chen, "Ikk β is an irf5 kinase that instigates inflammation," *Proceedings of the National Academy of Sciences*, vol. 111, no. 49, pp. 17 438–17 443, 2014.
- [235] B. Bergström, M. H. Aune, J. A. Awuh, J. F. Kojen, K. J. Blix, L. Ryan, T. H. Flo, T. E. Mollnes, T. Espevik, and J. Stenvik, "Tlr8 senses staphylococcus aureus rna in human primary monocytes and macrophages and induces ifn- β production via a tak1–ikk β –irf5 signaling pathway," *The Journal of Immunology*, vol. 195, no. 3, pp. 1100–1111, 2015.
- [236] C. del Fresno, D. Soulat, S. Roth, K. Blazek, I. Udalova, D. Sancho, J. Ruland, and C. Ardavin, "Interferon- β production via dectin-1-syk-irf5 signaling in dendritic cells is crucial for immunity to c. albicans," *Immunity*, vol. 38, no. 6, pp. 1176–1186, 2013.
- [237] M. B. Kursu, W. R. Rudnicki *et al.*, "Feature selection with the boruta package," *J Stat Softw*, vol. 36, no. 11, pp. 1–13, 2010.
- [238] R. Manetti, P. Parronchi, M. G. Giudizi, M. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani, "Natural killer cell stimulatory factor (interleukin 12 [il-12]) induces t helper type 1 (th1)-specific immune responses and inhibits the development of il-4-producing th cells." *The Journal of experimental medicine*, vol. 177, no. 4, pp. 1199–1204, 1993.
- [239] M. D. Kraaij, E. J. Vereyken, P. J. Leenen, T. P. van den Bosch, F. Rezaee, M. G. Betjes, C. C. Baan, and A. T. Rowshani, "Human monocytes produce interferon-gamma upon stimulation with lps," *Cytokine*, vol. 67, no. 1, pp. 7–12, 2014.
- [240] U. Grohmann, M. L. Belladonna, C. Vacca, R. Bianchi, F. Fallarino, C. Orabona, M. C. Fioretti, and P. Puccetti, "Positive regulatory role of il-12 in macrophages and modulation by ifn- γ ," *The Journal of Immunology*, vol. 167, no. 1, pp. 221–227, 2001.

- [241] A. Dobin and T. R. Gingeras, "Mapping rna-seq reads with star," *Current protocols in bioinformatics*, vol. 51, no. 1, pp. 11–14, 2015.
- [242] M. B. Bracken, "Why animal studies are often poor predictors of human reactions to exposure," *Journal of the Royal Society of Medicine*, vol. 102, no. 3, pp. 120–122, 2009.
- [243] M. M. Wurfel, W. Y. Park, F. Radella, J. Ruzinski, A. Sandstrom, J. Strout, R. E. Bumgarner, and T. R. Martin, "Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity," *The Journal of Immunology*, vol. 175, no. 4, pp. 2570–2578, 2005.
- [244] D. Wu, T. L. Dinh, B. P. Bausk, and D. R. Walt, "Long-term measurements of human inflammatory cytokines reveal complex baseline variations between individuals," *The American Journal of Pathology*, vol. 187, no. 12, pp. 2620–2626, 2017.
- [245] D. Yeung, S. Ciotti, S. Purushothama, E. Gharakhani, G. Kuesters, B. Schlain, C. Shen, D. Donaldson, and A. Mikulskis, "Evaluation of highly sensitive immunoassay technologies for quantitative measurements of sub-pg/ml levels of cytokines in human serum," *Journal of immunological methods*, vol. 437, pp. 53–63, 2016.
- [246] M. H. Veldman-Jones, R. Brant, C. Rooney, C. Geh, H. Emery, C. G. Harbron, M. Wappett, A. Sharpe, M. Dymond, J. C. Barrett *et al.*, "Evaluating robustness and sensitivity of the nanostring technologies ncounter platform to enable multiplexed gene expression analysis of clinical samples," *Cancer research*, vol. 75, no. 13, pp. 2587–2593, 2015.
- [247] G. Bondar, W. Xu, D. Elashoff, X. Li, E. Faure-Kumar, T.-M. Bao, T. Grogan, J. Moose, and M. C. Deng, "Comparing ngs and nanostring platforms in peripheral blood mononuclear cell transcriptome profiling for advanced heart failure biomarker development," *Journal of Biological Methods*, vol. 7, no. 1, 2020.
- [248] M.-L. Decker, V. Gotta, S. Wellmann, and N. Ritz, "Cytokine profiling in healthy children shows association of age with cytokine concentrations," *Scientific reports*, vol. 7, no. 1, pp. 1–10, 2017.

- [249] T. R. Kollmann, O. Levy, R. R. Montgomery, and S. Goriely, "Innate immune function by toll-like receptors: distinct responses in newborns and the elderly," *Immunity*, vol. 37, no. 5, pp. 771–783, 2012.
- [250] J.-C. Bambou, A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau, "In vitro and ex vivo activation of the tlr5 signaling pathway in intestinal epithelial cells by a commensal escherichia coli strain," *Journal of biological chemistry*, vol. 279, no. 41, pp. 42 984–42 992, 2004.
- [251] I. Hoogland, D. Westhoff, J.-Y. Engelen-Lee, J. Melief, M. Valls Serón, J. H. Houben-Weerts, I. Huitinga, D. J. Van Westerloo, T. van der Poll, W. A. van Gool *et al.*, "Microglial activation after systemic stimulation with lipopolysaccharide and escherichia coli," *Frontiers in cellular neuroscience*, vol. 12, p. 110, 2018.
- [252] O. M. Pena, D. G. Hancock, N. H. Lyle, A. Linder, J. A. Russell, J. Xia, C. D. Fjell, J. H. Boyd, and R. E. Hancock, "An endotoxin tolerance signature predicts sepsis and organ dysfunction at initial clinical presentation," *EBioMedicine*, vol. 1, no. 1, pp. 64–71, 2014.
- [253] M. Kox, S. de Kleijn, J. C. Pompe, B. P. Ramakers, M. G. Netea, J. G. van der Hoeven, C. W. Hoedemaekers, and P. Pickkers, "Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia," *Critical care medicine*, vol. 39, no. 8, pp. 1866–1870, 2011.
- [254] N. Rittig, H. H. Thomsen, E. Bach, J. O. L. Jørgensen, and N. Møller, "Hormone and cytokine responses to repeated endotoxin exposures—no evidence of endotoxin tolerance after 5 weeks in humans," *Shock*, vol. 44, no. 1, pp. 32–35, 2015.
- [255] S. K. Biswas and E. Lopez-Collazo, "Endotoxin tolerance: new mechanisms, molecules and clinical significance," *Trends in immunology*, vol. 30, no. 10, pp. 475–487, 2009.
- [256] G. Monneret, F. Venet, A. Pachot, and A. Lepape, "Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony," *Molecular medicine*, vol. 14, no. 1, pp. 64–78, 2008.
- [257] M. Sarasin-Filipowicz, E. J. Oakeley, F. H. Duong, V. Christen, L. Terracciano, W. Filipowicz, and M. H. Heim, "Interferon signaling and treatment outcome

- in chronic hepatitis c,” *Proceedings of the National Academy of Sciences*, vol. 105, no. 19, pp. 7034–7039, 2008.
- [258] S. Wang, Y. Li, Y. Shen, J. Wu, Y. Gao, S. Zhang, L. Shao, J. Jin, Y. Zhang, and W. Zhang, “Screening and identification of a six-cytokine biosignature for detecting tb infection and discriminating active from latent tb,” *Journal of translational medicine*, vol. 16, no. 1, p. 206, 2018.
- [259] M. M. Davis, C. M. Tato, and D. Furman, “Systems immunology: just getting started,” *Nature immunology*, vol. 18, no. 7, p. 725, 2017.
- [260] A.-C. Villani, S. Sarkizova, and N. Hacohen, “Systems immunology: Learning the rules of the immune system,” *Annual review of immunology*, vol. 36, pp. 813–842, 2018.
- [261] Y. Li, M. Oosting, P. Deelen, I. Ricaño-Ponce, S. Smeekens, M. Jaeger, V. Matzaraki, M. A. Swertz, R. J. Xavier, L. Franke *et al.*, “Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi,” *Nature medicine*, vol. 22, no. 8, pp. 952–960, 2016.
- [262] V. E. Villegas and P. G. Zaphiropoulos, “Neighboring gene regulation by antisense long non-coding rnas,” *International journal of molecular sciences*, vol. 16, no. 2, pp. 3251–3266, 2015.
- [263] S. Schnyder-Candrian, R. M. Strieter, S. L. Kunkel, and A. Walz, “Interferon- α and interferon- γ down-regulate the production of interleukin-8 and ena-78 in human monocytes,” *Journal of leukocyte biology*, vol. 57, no. 6, pp. 929–935, 1995.
- [264] N. Molnarfi, L. Gruaz, J.-M. Dayer, and D. Burger, “Opposite effects of ifn β on cytokine homeostasis in lps-and t cell contact-activated human monocytes,” *Journal of neuroimmunology*, vol. 146, no. 1-2, pp. 76–83, 2004.
- [265] H. FUJISAWA, B. WANG, D. N. SAUDER, and S. KONDO, “Effects of interferons on the production of interleukin-6 and interleukin-8 in human keratinocytes,” *Journal of interferon & cytokine research*, vol. 17, no. 6, pp. 347–353, 1997.
- [266] F. Mattei, G. Schiavoni, F. Belardelli, and D. F. Tough, “Il-15 is expressed by dendritic cells in response to type i ifn, double-stranded rna, or lipopolysaccharide and

- promotes dendritic cell activation," *The Journal of Immunology*, vol. 167, no. 3, pp. 1179–1187, 2001.
- [267] D. Azoulay, K. Mausner-Fainberg, N. Urshansky, F. Fahoum, and A. Karni, "Interferon- β therapy up-regulates bdnf secretion from pbmcs of ms patients through a cd40-dependent mechanism," *Journal of neuroimmunology*, vol. 211, no. 1-2, pp. 114–119, 2009.
- [268] K. S. Kumar, W. Tang, A. K. Ravindranath, W. A. Clark, E. Croze, and S. Y. Fuchs, "Scfhos ubiquitin ligase mediates the ligand-induced down-regulation of the interferon- α receptor," *The EMBO journal*, vol. 22, no. 20, pp. 5480–5490, 2003.
- [269] H. Zheng, J. Qian, C. J. Carbone, N. A. Leu, D. P. Baker, and S. Y. Fuchs, "Vascular endothelial growth factor-induced elimination of the type 1 interferon receptor is required for efficient angiogenesis," *Blood, The Journal of the American Society of Hematology*, vol. 118, no. 14, pp. 4003–4006, 2011.
- [270] Z. Ma, H. Qin, and E. N. Benveniste, "Transcriptional suppression of matrix metalloproteinase-9 gene expression by ifn- γ and ifn- β : critical role of stat-1 α ," *The Journal of Immunology*, vol. 167, no. 9, pp. 5150–5159, 2001.
- [271] P. J. Lupardus and K. C. Garcia, "The structure of interleukin-23 reveals the molecular basis of p40 subunit sharing with interleukin-12," *Journal of molecular biology*, vol. 382, no. 4, pp. 931–941, 2008.
- [272] A. Snijders, C. Hilkens, T. Van der Pouw Kraan, M. Engel, L. A. Aarden, and M. L. Kapsenberg, "Regulation of bioactive il-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit." *The Journal of Immunology*, vol. 156, no. 3, pp. 1207–1212, 1996.
- [273] C. Vasu, A. Wang, S. R. Gorla, S. Kaithamana, B. S. Prabhakar, and M. J. Holterman, "Cd80 and cd86 c domains play an important role in receptor binding and co-stimulatory properties," *International immunology*, vol. 15, no. 2, pp. 167–175, 2003.
- [274] G. Monneret, A. Lepape, N. Voirin, J. Bohé, F. Venet, A.-L. Debard, H. Thizy, J. Bienvenu, F. Gueyffier, and P. Vanhems, "Persisting low monocyte human

- leukocyte antigen-dr expression predicts mortality in septic shock,” *Intensive care medicine*, vol. 32, no. 8, pp. 1175–1183, 2006.
- [275] J. Manjuck, D. C. Saha, M. Astiz, L.-J. Eales, and E. C. Rackow, “Decreased response to recall antigens is associated with depressed costimulatory receptor expression in septic critically ill patients,” *Journal of Laboratory and Clinical Medicine*, vol. 135, no. 2, pp. 153–160, 2000.
- [276] A. Piani, J. P. Hossle, T. Birchler, C.-A. Siegrist, D. Heumann, G. Davies, S. Loeliger, R. Seger, and R. P. Lauener, “Expression of mhc class ii molecules contributes to lipopolysaccharide responsiveness,” *European journal of immunology*, vol. 30, no. 11, pp. 3140–3146, 2000.
- [277] M. Astiz, D. Saha, D. Lustbader, R. Lin, and E. Rackow, “Monocyte response to bacterial toxins, expression of cell surface receptors, and release of anti-inflammatory cytokines during sepsis,” *Journal of Laboratory and Clinical Medicine*, vol. 128, no. 6, pp. 594–600, 1996.
- [278] E. Wiesemann, M. Deb, C. Trebst, B. Hemmer, M. Stangel, and A. Windhagen, “Effects of interferon- β on co-signaling molecules: upregulation of cd40, cd86 and pd-l2 on monocytes in relation to clinical response to interferon- β treatment in patients with multiple sclerosis,” *Multiple Sclerosis Journal*, vol. 14, no. 2, pp. 166–176, 2008.
- [279] M. Fujihara, T. Takahashi, M. Azuma, C. Ogiso, T. Maekawa, H. Yagita, K. Okumura, and S. Sekiguchi, “Decreased inducible expression of cd80 and cd86 in human monocytes after ultraviolet-b irradiation: its involvement in inactivation of allogeneicity,” 1996.
- [280] J.-H. Yen, W. Kong, K. M. Hooper, F. Emig, K. M. Rahbari, P.-C. Kuo, B. A. Scofield, and D. Ganea, “Differential effects of ifn- β on il-12, il-23, and il-10 expression in tlr-stimulated dendritic cells,” *Journal of leukocyte biology*, vol. 98, no. 5, pp. 689–702, 2015.
- [281] R. Rutkowski, T. Moniuszko, A. Stasiak-Barmuta, B. Kosztyla-Hojna, M. Alifier, K. Rutkowski, and A. Tatarczuk-Krawiel, “Cd80 and cd86 expression on lps-stimulated monocytes and the effect of cd80 and cd86 blockade on il-4 and ifn-

- gamma production in nanotopic bronchial asthma,” *Archivum Immunologiae et Therapiae Experimentalis-English Edition*-, vol. 51, no. 6, pp. 421–428, 2003.
- [282] B. L. McRae, R. T. Semnani, M. P. Hayes, and G. A. van Seventer, “Type i ifns inhibit human dendritic cell il-12 production and th1 cell development,” *The Journal of Immunology*, vol. 160, no. 9, pp. 4298–4304, 1998.
- [283] E. Bartholome, F. Willems, A. Crusiaux, K. Thielemans, L. Schandené, and M. Goldman, “Interferon-beta inhibits th1 responses at the dendritic cell level. relevance to multiple sclerosis.” *Acta neurologica belgica*, vol. 99, no. 1, pp. 44–52, 1999.
- [284] J. M. van Seventer, T. Nagai, and G. A. van Seventer, “Interferon- β differentially regulates expression of the il-12 family members p35, p40, p19 and ebi3 in activated human dendritic cells,” *Journal of neuroimmunology*, vol. 133, no. 1-2, pp. 60–71, 2002.
- [285] H. Heystek, B. Den Drijver, M. Kapsenberg, R. van Lier, and E. De Jong, “Type i ifns differentially modulate il-12p70 production by human dendritic cells depending on the maturation status of the cells and counteract ifn- γ -mediated signaling,” *Clinical Immunology*, vol. 107, no. 3, pp. 170–177, 2003.
- [286] A. A. Byrnes, X. Ma, P. Cuomo, K. Park, L. Wahl, S. F. Wolf, H. Zhou, G. Trinchieri, and C. L. Karp, “Type i interferons and il-12: convergence and cross-regulation among mediators of cellular immunity,” *European journal of immunology*, vol. 31, no. 7, pp. 2026–2034, 2001.
- [287] R. A. de Paus, A. van Wengen, I. Schmidt, M. Visser, E. M. Verdegaal, J. T. van Dissel, and E. van de Vosse, “Inhibition of the type i immune responses of human monocytes by ifn- α and ifn- β ,” *Cytokine*, vol. 61, no. 2, pp. 645–655, 2013.
- [288] M. Hasan, B. Beitz, V. Rouilly, V. Libri, A. Urrutia, D. Duffy, L. Cassard, J. P. Di Santo, E. Mottez, L. Quintana-Murci *et al.*, “Semi-automated and standardized cytometric procedures for multi-panel and multi-parametric whole blood immunophenotyping,” *Clinical Immunology*, vol. 157, no. 2, pp. 261–276, 2015.
- [289] U. Grömping *et al.*, “Relative importance for linear regression in r: the package relaimpo,” *Journal of statistical software*, vol. 17, no. 1, pp. 1–27, 2006.