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HIGHLIGHTS

Basic

REVIEW

Whole blood stimulation as a tool for studying the human immune system

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The human immune system is best accessible via tissues and organs not requiring major surgical intervention, such as blood. In many circumstances, circulating immune cells correlate with an individual's health state and give insight into physiological and pathophysiological processes. Stimulating whole blood *ex vivo* is a powerful tool to investigate immune responses. In the context of clinical research, the applications of whole blood stimulation include host immunity, disease characterization, diagnosis, treatment, and drug development. Here, we summarize different setups and readouts of whole blood assays and discuss applications for preclinical research and clinical practice. Finally, we propose combining whole blood stimulation with high-throughput technologies, such as single-cell RNA-sequencing, to comprehensively analyze the human immune system for the identification of biomarkers, therapeutic interventions as well as companion diagnostics.

Keywords: Whole blood · Ex vivo stimulation · Functional immune assay · Precision medicine

Introduction

The immune system and its cellular components are involved in many homeostatic functions of all tissues and organs, while lymph and blood vessels are systemic connectors of the different parts of this complex, distributed organ system. During stress situations, in particular, diseases ranging from infections to cancer and neurodegeneration, mechanisms of specific and nonspecific immune activation are hallmarks of how the immune system reacts to such insults. Most of the major cellular components and molecular mechanisms of the immune system have been identified and characterized in murine systems. During the last decades, it has become increasingly evident that the human immune system requires specific attention since many known aspects derived from animal studies do not easily translate. As many experimental approaches are not applicable to humans, sophisticated surrogate *ex vivo* systems are required to study the compartments of the human immune system. Combined with advances in omics technologies and the data sciences, scientists now have the tools in hand to comprehensively assess the molecular components and dynamics of human immune cells in high parameter space, as well as the analysis approaches including computational modeling and

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machine learning to generate insights from such big data and to complement existing standard immunological methods. Here, we describe the potential of whole blood stimulation assays combined with high-dimensional single-cell level data acquisition to reach a detailed understanding of human immunity and how the immune system contributes to disease trajectories. We also discuss how such assays are utilized for diagnostic purposes and might guide treatment and drug development in the future. In this review, we focus on the usage of peripheral blood to study the immune response as it is the most accessible sample in the clinics, while the importance of other lymphoid organs and immunological tissue sites have been discussed elsewhere [1].

Ex vivo stimulation to understand the immune system

Stimulation studies can be carried out to gain knowledge about the function of the immune system. In contrast to inbred mouse strains, there is a high interindividual variation in the human immune response due to genetic and environmental factors such as diet, previous infections, or vaccinations. This variance in the immune response is often not visible at a steady state, as the full spectrum of the immune response manifests only upon activation as induced by infection or disease. The effects of genetic variations such as single nucleotide polymorphisms are classically assessed by linking them to variations in gene expression [2, 3]. For example, stimulation with ligands of viral and bacterial origin has revealed different patterns of gene expression linked to alternative genotypes in human monocytes and dendritic cells [4, 5]. Larger consortia efforts, such as the Human Functional Genomics Project [6, 7] and the Milieu Intérieur project [8] have set out to comprehensively investigate how host and environmental factors influence the immune response of healthy individuals on the population level at steady state as well as upon *ex vivo* stimulation. This approach has also been extended to assess vaccination-induced immunity [9, 10] and to identify disease-specific responses [11]. However, the knowledge of the nature of this interindividual variation of the immune response is still incomplete and is a prerequisite to better understanding differences in individual susceptibility to infectious diseases, the development of chronic inflammatory diseases, or autoimmunity.

Why to analyze whole blood?

Despite the fact that most immune responses occur at tissue sites, peripheral blood can harbor important information about systemic immune reactions and can be used to obtain insights into an individual's health status [6–8]. Peripheral blood contains the circulating immune cells migrating to and from tissues and organs and therefore correlates in many cases with immunological processes happening in the periphery. In some instances, it has been shown to serve as a surrogate for pathological reactions in diseased tissues and disease activity in the body [12]. For example, peripheral blood cells are indicative of local infections [13–15] and vaccination status [16]. Some studies support the notion that immunological alterations in the blood can be utilized to diagnose cancer [17, 18] and can be indicative of the treatment response [19, 20]. We recognize that for certain immunological reactions that are tissue-specific, tissue samples in form of needle biopsies or swabs might be necessary to capture this tissue-specific immune pathology [21]. However, to study the function of the human immune system, the usage of peripheral blood is widely applied since it is easily accessible from the clinics, allows longitudinal sampling, and scales in large clinical cohorts. From human blood, most frequently peripheral blood mononuclear cells (PBMCs) are isolated using density gradient centrifugation to separate mononuclear leukocytes from erythrocytes, granulocytes, and platelets. A major advantage of PBMCs is the possibility of cryopreservation in batches and consequent collective analysis at a later time point. The main aspects to consider when performing assays with whole blood, PBMCs, or isolated cell types are summarized in Table 1. Studies on cytokine secretion demonstrated that interindividual variation is better conserved when analyzing the production of monocytic cytokines such as TNFα and IL-6 from stimulated PBMCs compared with isolated and *in vitro* differentiated cells such as monocyte-derived macrophages [7]. Similarly, cytokine production from whole blood over an 8-week sampling time proved to be reproducible with smaller intraindividual than interindividual variation [22]. To further improve the reproducibility of whole blood assays, a user-friendly and highly standardized whole blood stimulation system (TruCulture) was applied in a multicenter clinical study assessing the release of 55 soluble proteins. This study revealed that the TruCulture system had less variability due to technical improvements and as a consequence higher reproducibility compared with PBMC stimulation [23].

In contrast to PBMCs, whole blood still contains all host serum factors as well as erythrocytes, platelets, and granulocytes representing a more physiological environment and allowing cell-to-cell communication during the time of stimulation [24]. Neutrophils are the most abundant leukocytes in the blood and are involved in various diseases including cancer, cardiovascular, neurodegenerative, autoimmune, and infectious diseases such as COVID-19 [25, 26]. Neutrophils have long been neglected in part due to technical challenges linked to their high fragility and sensitivity to cell death, but also since the complexity of their numerous functions in the context of immune homeostasis, during immune responses, and in disease pathophysiology have been overlooked [27].

Often certain immune cell types are isolated in high purity from peripheral blood prior to further analysis, which may be necessary for analysis of low frequency cell types such as dendritic cells. However, cell isolations are not only time-intensive but often induce unwanted preactivation of the cells, which leads to systematic alterations when analyzing the reactivity of immune cells. In contrast, the minimal number of preprocessing steps for whole blood allows closer comparability to the physiological *in vivo* state. Further, for some applications, the usage of whole blood has

Table 1. Comparison of the usage of whole blood, PBMCs, and isolated cell types in stimulation assays.

	Whole blood assays	PBMC assays	Isolated cell-type assays
Cell types	All blood cell types (including granulocytes, erythrocytes, and platelets)	PBMC populations	Selected cell type depending on the isolation protocol
Cell numbers and composition	Adjustment of total cell number by blood volume used	Total PBMC number adjustable	Exact adjustment of cell number possible
	Immanent interindividual variation in cell type proportions	Immanent interindividual variation in cell type proportions in PBMC fraction	-
	High percentage of neutrophils, low number of smaller or rare populations	High percentage of T cells	Isolation and enrichment of rare cell populations possible
(Cellular) interaction	Interactions between all blood cell types possible	Interactions between PBMCs possible	Interaction only among cells of one cell type (isolated environment)
	Interaction with serum factors (e.g. serum antibodies) possible	-	-
	Direct response to a stimulus or indirect response via signals derived from primary responding cell types; cell type specificity depending on readout	Direct response to a stimulus or indirect response via signals derived from primary responding cell types; cell type specificity depending on the readout	Cell type-specific response to a stimulus
Isolation process	Immediate start of assay possible	Isolation procedure delays start of assay and exposes cells to nonphysiological environment before the assay	Isolation procedure delays start of assay and exposes cells to nonphysiological environment before the assay
	No/minimal processing steps before assay, reducing risk of preactivation due to handling; fragile granulocytes require extra careful handling of blood to avoid degranulation	Cells might get preactivated through isolation process before assay Isolation protocol can introduce unphysiological bias in cell type composition	Cells might get preactivated through isolation process before assay
Storage	No cryopreservation of functional and viable granulocytes possible, immediate processing necessary	Cryopreservation and long-term storage possible	Cryopreservation and long-term storage possible depending on the cell type
Experimental batches	The need for freshly drawn blood requires careful scheduling of blood donors across experimental groups to create interpretable batches of the stimulation as well as of downstream readouts conducted with viable cells	Frozen cells from multiple isolation procedures/multiple donors thawed at the same time can be used together for the assay	Isolation procedure requiring fresh samples needs careful scheduling of blood donors across experimental groups to create interpretable batches of the stimulation as well as downstream readouts conducted with viable, not cryopreservable cells; isolated cells that can be cryopreserved may be pooled for analysis
Physiological relevance	Closest to *in vivo* setting	Good representation of lymphocyte and monocyte compartment but more artificial soluble environment	Artificial setting with one isolated cell type

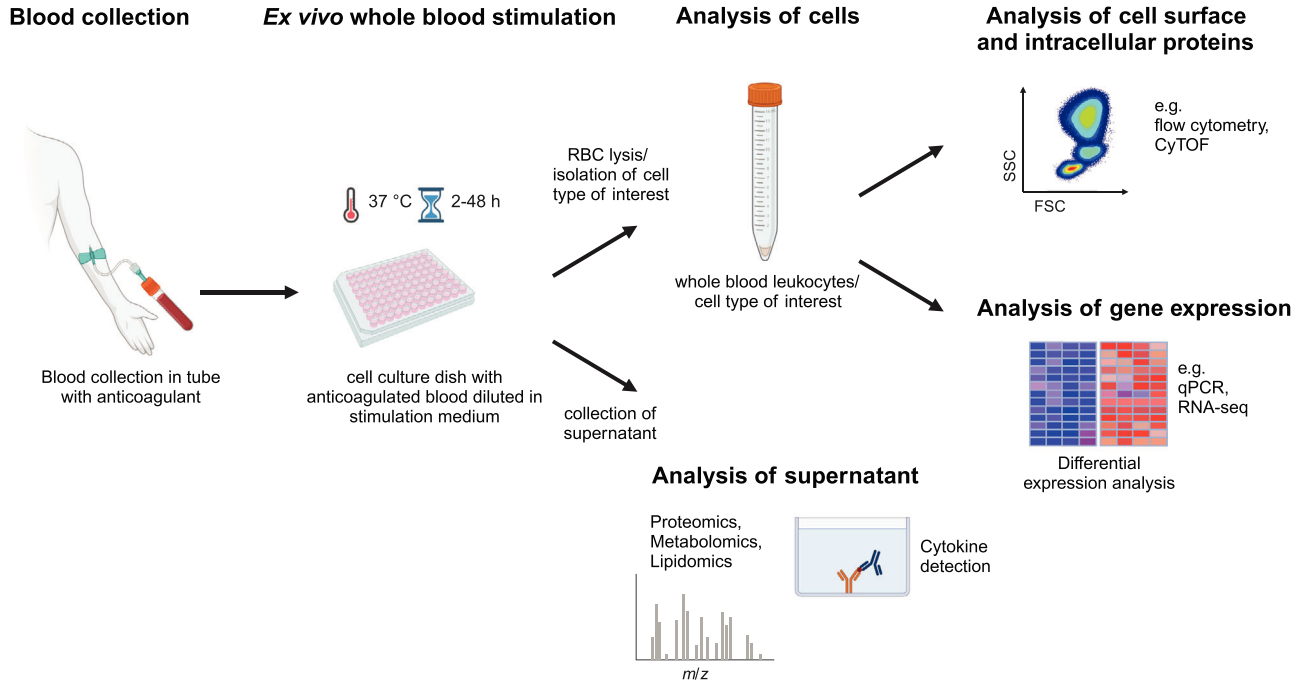

Figure 1. Setup of whole blood stimulation experiments. Blood is collected in tubes containing anticoagulants and distributed into a cell culture plate (e.g. 24, 48, or 96 well) or in polypropylene tubes containing a cell culture medium with or without stimuli. Addition of antibiotics or supplements is optional. Blood is diluted with the stimulation medium at a ratio of 1:2 to 1:10. The whole blood stimulation is performed at 37°C for the time of choice (e.g. 2–48 h). After the incubation, the cell culture supernatant can be collected for the analysis of secreted proteins, metabolites, and lipids. The residual blood cells are collected and a red blood cell lysis is performed to retrieve whole blood leukocytes. Alternatively, a cell type of interest or PBMCs can be isolated or for analysis of gene expression a whole blood RNA extraction approach can be applied. The cell surface and intracellular protein expression can be examined by flow cytometry. Gene expression can be investigated by qPCR, targeted or whole transcriptome RNA-seq, or other gene expression analysis approaches. Figure created with Biorender.com.

additional practical advantages as results can be obtained from only a few milliliters of blood without the need to isolate cells first. For example, T-cell immunity in the context of SARS-CoV-2 infection can be quantified by direct qPCR-based rapid T-cell activation assay directly from whole blood [28]. Taken together, performing functional immune assays with whole blood draws a more complete picture of an individual's immune state by including all present immune cells in the blood and soluble host factors.

Whole blood assays

Whole blood assays can be performed in different formats and with different readouts. In many studies, anticoagulated blood is diluted with cell culture medium containing a stimulus of choice and incubated for defined time periods [22, 28–30] (Fig. 1). For many applications, cell culture medium without serum as a supplement is sufficient as whole blood contains serum factors and is nutrient-rich. While different anticoagulants (e.g. EDTA or heparin) have been successfully used, and are impacting particularly Calcium-sensitive functional assays, the best choice for generating high-dimensional data, such as transcriptome data, for all blood cell types remains to be further investigated [31–33]. Blood collection time, delayed sample handling, or storage temperature can also influence the functionality of blood leukocytes [32, 34, 35]. Prolonged storage or cooling of samples, and thus deviations from their *in vivo* state, impacts cell reactivity in functional assays, particularly in reactive cells such as neutrophils [32].

To overcome these limitations, the TruCulture system, a whole blood syringe-based assay system, has been introduced where the blood is directly drawn into a blood collection tube containing a stimulation medium to perform standardized immunophenotyping [29]. Alternatively, anticoagulated blood can be distributed into tissue culture plates containing a stimulation medium. Stimulation times vary from 2–48 h across studies depending on the stimulus and the readout used (Table 2). Whole blood stimulation assays are mostly limited to the early phases of responses as cells cannot be cultured longer than approximately 2 days without providing new nutrients and losing fragile, short-lived immune cells such as the granulocytes. The stimulus-triggered immune reaction may be investigated either by analyzing the cell's secretome in the supernatant (antibody-based assays, mass spectrometry) or by characterizing the immune cells themselves, that is, their surface and intracellular proteome (by flow cytometry or CyTOF) and transcriptome (by RNA-sequencing, qPCR) (Fig. 1). So far, the majority of studies focused on the analysis of soluble ligands such as cytokines and chemokines in the culture supernatant [7, 29]. For gene expression analysis, RNA may either be directly extracted from whole blood or from whole blood leukocytes. The transcriptome can be analyzed with targeted gene expression

Table 2. Selected studies applying whole blood assays.

Setup	Stimulation time (h)	Readout	References
TruCulture tubes, heparin, 1 mL peripheral blood, diluted 1:3	22	Protein measurements in supernatant	Duffy et al. [29] Duffy et al. [23]
48-well plate, heparin, 100 μL peripheral blood, diluted 1:5	48	Protein measurements in supernatant	Li et al. [7]
96-well plate, heparin, cord blood, diluted 1:2	18	Metabolomics, lipidomics	Diray-Arce et al. [45]
15 mL tubes, 400 μL peripheral blood, diluted 1:2 with medium containing antibiotics	24	Intracellular cytokine production by flow cytometry analysis	Corbière et al. [44]
2 mL 96-well plate, citrate-based anticoagulant, 500 μL peripheral blood, diluted 1:2	6	Protein measurements in supernatant, flow cytometry, gene expression analysis (microarray)	Cepika et al. [37]
TruCulture tubes, heparin, 1 mL peripheral blood, diluted 1:3	22	Gene expression analysis (Nanostring)	Piasecka et al. [36] Smith et al. [59]
Heparin, 500 μL peripheral blood, diluted 1:2	2	Gene expression analysis (microarray)	Alsina et al. [57]
TruCulture tubes, heparin, 1 mL peripheral blood, diluted 1:3	30	Bulk RNA-sequencing	Stein et al. [38]

Note: The table displays exemplary studies applying whole blood stimulation assays with regard to the assay setup (anticoagulant, cell culture dish, dilution factor), stimulation time, and readout.

analysis approaches [36, 37] or whole transcriptome RNA-sequencing [38] for comprehensive analysis. Of note, small volumes of blood, such as 100 μL, may be used for transcriptome readouts, which is especially of advantage in clinical studies, where volume of blood is limited.

Moreover, staining of surface markers by flow cytometry may be performed before [37] or after erythrocyte lysis [39, 40]. For example, whole blood intracellular cytokine stainings were used to assess antigen-specific T-cell responses in a robust and reproducible way that may also be applied to study vaccine efficacy [41–44].

For studying metabolic responses to stimuli, mass spectrometry-based metabolomics may be utilized to analyze the secreted metabolites in the supernatant [45, 46]. This can be further combined with SCENITH, a flow cytometry-based method for studying cell-specific metabolic changes that is compatible with whole blood [47]. As immune responses are multifactorial, combining different readouts downstream of whole blood assays may give the most insights into the cellular and molecular mechanisms operative during an immune response.

Clinical applications of whole blood stimulation

In the context of translational or clinical research questions, whole blood stimulation assays provide the opportunity to study perturbation effects *ex vivo* in a safe and controlled manner. This creates insights into immune responses to stimuli that cannot be safely administered to humans *in vivo* or into unforeseeable processes, such as acute infections, for which it is difficult to collect samples at early time points as well as a matched baseline sample.

Human whole blood stimulation assays are very versatile as they are based on two components that come with great variety — whole blood of humans and a stimulus (or stimuli combination) (Fig. 2). The blood can be taken from healthy individuals, patients with acute or chronic diseases or individuals with a certain medical history, be it a prior infection or another *in vivo* immunological intervention, such as a treatment, vaccination or surgery. Also, the list of available stimuli is endless, comprising cytokines and other secreted mediators, drugs and immunotherapeutics, whole microbes, components, or antigens of viral or bacterial origin (e.g. LPS, SEB, SARS-CoV-2 spike protein), allergens as well as commonly used agonistic compounds (polyI:C, R848, CpG, anti-CD3/28 antibodies). Depending on the complexity of the readout, two central questions can be approached. First, the magnitude of the stimulation response and second, the qualitative response, i.e. which pathways and signals are modulated (Fig. 2). Especially to estimate qualitative differences, the application of comprehensive, nontargeted readouts gives a more complete picture of the response. Thus, whole blood stimulation assays have a wide variety of applications in clinical research, covering disease characterization, host immunity, diagnosis, treatment, and drug development (Fig. 2). In the following section, we will provide a more detailed view of different application scenarios in selected studies.

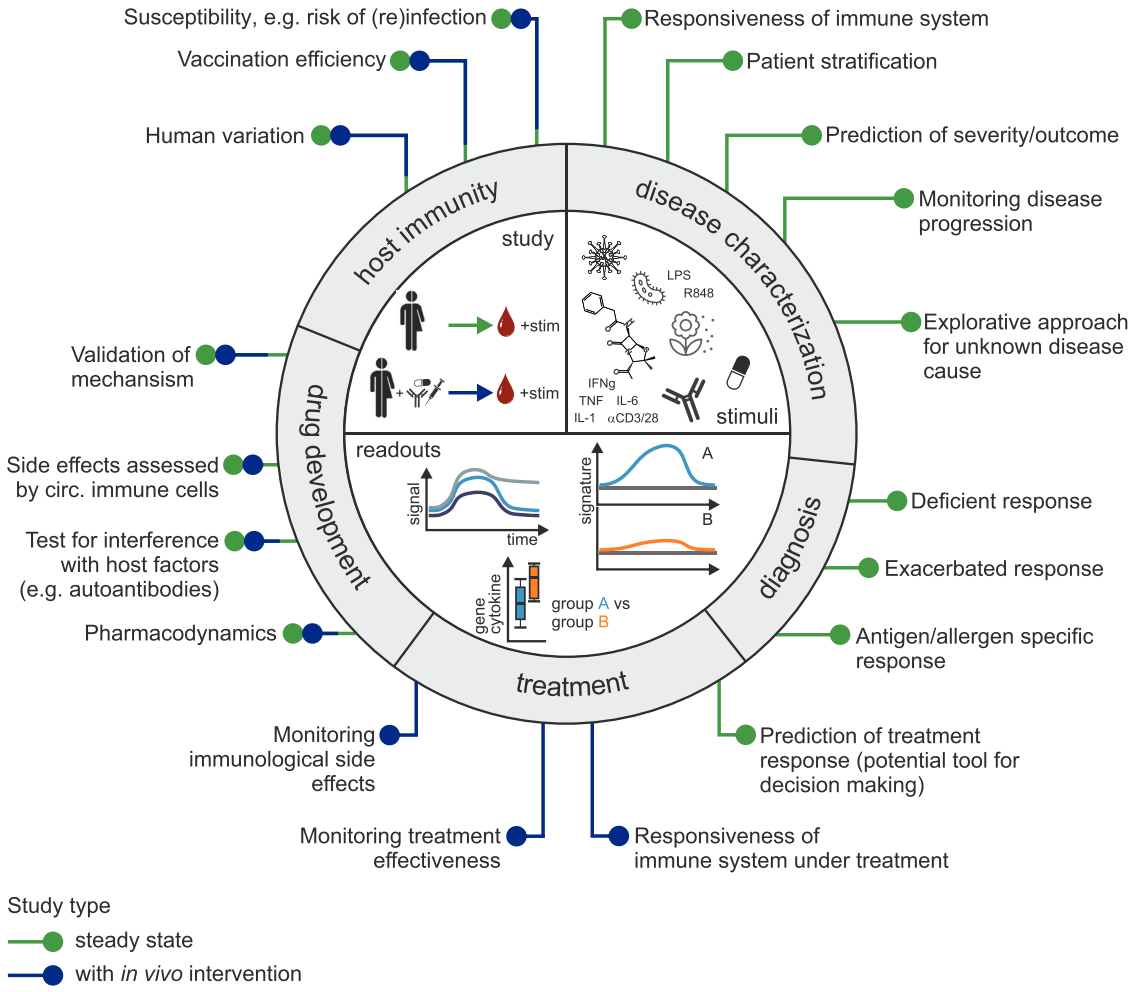

Figure 2. Application of whole blood stimulation in clinical and translational research. Human whole blood stimulation comprises two main components: blood and a stimulus (or stimuli combination), as conceptualized in the central circle of the figure. Blood is collected from healthy individuals or patients at steady state (without *in vivo* intervention, green) or individuals that underwent an *in vivo* intervention such as vaccination or treatment (blue) shown in the upper left panel. A variety of stimuli can be applied ranging from synthetic compounds to whole microbes (upper right). The readouts can be qualitative, quantitative, or longitudinal (lower panel). The outer circle displays potential applications in clinical research addressing disease pathology, treatment, diagnosis, drug development, and host immunity. Each application has different application scenarios as indicated by the bullet points surrounding the circle, which can be performed with blood from donors with (blue dot), without in vivo intervention (green dot), or both (green and blue dot). Parts of the figure contain elements from Colourbox.de.

Host immunity

Host and environmental factors such as age, sex, genetics, diet, and lifestyle choices account for interindividual variance in the immune response. The influence of these factors on host immunity may be studied with whole blood stimulation assays. Characterization of the "normal and healthy" immune response to stimuli is fundamental to understanding deviations from this response, which can be linked to infection and other disease susceptibilities. To study host immunity, it is especially important to rely on large enough, well-defined cohorts that allow investigation of intergroup and interindividual differences, for example, tackled by projects like the Milieu Intérieur project and the Human Functional Genomics Project [6–8]. Genetic variation such as single nucleotide polymorphisms is one of multiple factors which can introduce variance in the immune response and can be studied with whole blood assays [48, 49]. For example, stimulation with influenza A virus but not with LPS and polyI:C revealed a differential transcriptional response in Rhesus-negative male individuals [50]. Another frequently used application of whole blood assays is to investigate host immunity in the context of vaccination. IFN-γ release assays were used to study immunity to vaccines by stimulating whole blood with a vaccine-specific or pathogen-specific peptide pool to compare immunity induced by combinations of vaccines against SARS-CoV-2 [51, 52]. This approach has been extended by additional cytokines as readouts to test the effect of different vaccine formulations [53, 54]. In addition to the readout of soluble mediators, cell-mediated immunity after vaccination is assessed with recall assays, which are applied to estimate the magnitude and duration of cell-

mediated immunity after vaccination in field studies [42, 55]. A study combining cytokine release and cellular marker measurements from a 2-day whole blood stimulation assay stratified SARS-CoV-2 vaccinated, COVID-19 convalescent, and healthy control individuals [56]. These methods are not limited to studying the adult immune system but can also be applied to study immunity in infants. For example, whole blood stimulation with cord blood and peripheral blood of newborns was used to study the response to the Bacille Calmette–Guérin vaccine in infants [45].

Disease characterization and treatment

Evaluation of responsiveness and functionality of the immune system as part of disease pathology is also central when studying acute and chronic diseases. Whole blood stimulation assays enable assessment of an altered immune response status that may not be apparent at steady state as well as of potential treatment responses *ex vivo*. The generated insights are of great interest as altered responsiveness to a stimulus can have important consequences for patients with regard to their ability to establish immunity and consequent protection against pathogens and their disease progression concerning severity, outcome, and treatment response.

Whole blood stimulation assays have been used to study immune responses in many disease conditions such as primary immunodeficiencies [57], autoimmune and autoinflammatory diseases [37, 58], and viral [59, 60] and bacterial [61] infections to name only a few. As such, clinically defined patient subgroups may be assessed, compared, and monitored. For example, such comparisons were investigated for active and latent tuberculosis infection [61], COVID-19 severity [59], and systemic juvenile arthritis in treated and untreated patients with differing disease activity [37]. Further, variance in response patterns in clinical cohorts can also enable new or improved subgroup stratifications. For example, combined interpretation of IFN-γ levels induced by parallel stimulation with *M. tuberculosis* antigen and live Bacillus Calmette–Guérin (BCG) improved the differentiation of active and latent tuberculosis [61]. First attempts were made to stratify sepsis patients based on the transcriptional *in vitro* response of a small panel of genes to LPS and SEB [62]. However, such targeted approaches need to be further validated — best with genome-wide assays — and the new subgroups evaluated for clinical impact.

Combining whole blood stimulation with longitudinal sampling of patients has been used to monitor changes in responsiveness over time with regard to disease progression and treatment-induced effects. For example, longitudinal sampling before and during treatment was performed to study innate immune function during different cancer treatments [63] and combinational antiretroviral therapy in HIV [64]. Further, longitudinally sampling COVID-19 patients from admission to the hospital until dismissal (or death) revealed that lower release of IL-1β upon LPS as well as IL-12 and IL-17A upon R848 stimulation at admission were associated with more severe disease at peak severity [65]. Besides prediction of disease progression and outcome, prediction of treatment response is another major question in precision medicine. Although not really studied yet for whole blood stimulation, patients with axial spondyloarthritis sampled before initialization of anti-TNF treatment were retrospectively stratified by their therapeutic response and subsequent comparison of their targeted transcriptomic profiles upon LPS and SEB stimulation identified genes associated with IL-1 signaling, leukocyte adhesion, and chemotaxis to be higher expressed in responders compared with nonresponders [66].

Suppressed immunity due to disease, immunosuppressive treatment, or severe shock syndrome elevates the risk of infection making monitoring of the global immune status in these patients crucial. For transplant patients, a cellular assay quantifying ATP production from $CD4^+$ cells post whole blood stimulation was developed to monitor immunosuppressive treatment regimens [67] which has additional potential to indicate risk of infection and transplant rejection [68]. Although many studies focus on measuring cytokine production upon whole blood stimulation, more recent studies also investigate transcriptional changes on bulk level. However, they mostly apply targeted transcriptomics approaches limiting the number of reported genes to a few hundred. Usage of genome-wide and cell type-specific readouts of whole blood stimulation assays would also allow the identification of unknown gene regulations and potentially new therapeutic targets. Rare examples of genome-wide measurements are reported for transcriptional regulation in response to multiple stimuli in patients with primary immunodeficiencies [57] or in systemic juvenile idiopathic arthritis, for which also intracellular and soluble cytokine production was assessed [37].

Diagnosis

If whole blood stimulation assays lead to disease-specific functional readouts, these tests could also serve for diagnosis. Indeed, a few whole blood stimulation assays have been introduced as diagnostic tests in clinical practice. Most prominently, IFN-γ release assays (e.g. QuantiFERON-TB Gold) are used to diagnose active and latent tuberculosis infection, measuring the IFN-γ production in whole blood upon stimulation with *M. tuberculosis*-derived peptides. First evaluated in a large cohort in 1998 [69], IFN-γ release assays have been frequently used in research and diagnostics [70]. Another example is the diagnosis of IgE-mediated allergies with basophil activation tests quantifying the proportion of activated basophils after *ex vivo* exposure of the blood to the allergen by flow cytometry [71, 72]. Introduced in 1994 [73], initial variations using whole blood stimulation followed soon after [74, 75]. Whole blood stimulation assays have also been developed for primary immunodeficiencies, where the missing activation of defective pathways is the readout. One example is chronic granulomatous disease [76], but other immunodeficiencies have also been considered [77], a field that needs further investigation.

Drug development

Whole blood stimulation assays are particularly promising approaches for immunomodulatory drug development, both at the preclinical stage as well as in phase 1 and 2 trials. The use of whole blood further bears the advantage that all host factors are present, potentially interfering with the drug's efficiency (e.g. drug-specific autoantibodies).

In preclinical studies, whole blood stimulation can be exploited to test the effect of a drug in blood as a human model system. Depending on the drug's properties, the drug itself is added to the blood alone [78] or an additional, activating stimulus is added simultaneously [79] or after preincubation of the drug [80]. Mechanisms of action as well as potential side effects of a drug can be determined by activating pathways upstream of a drug's target. Further, whole blood stimulation assays provide a model system to evaluate whether mechanisms studied *in vivo* in mouse systems are likely conserved in humans [79].

A typical application of *ex vivo* whole blood stimulation assays in phase I clinical trial settings is reading out the effectiveness of a particular drug to suppress for example the release of proinflammatory cytokines. This can be further coupled to longitudinal study designs, which then even provide information on *in vivo* pharmacodynamics, a scenario that has been described for single- as well as multidose treatments [80–83].

In a few cases, whole blood stimulation assays have also been reported to complement later-stage clinical development. For example, in a phase 2a trial a single dose of etokimab, an anti-IL33 antibody, was shown to strongly inhibit IFN-γ release after *ex vivo* IL12/IL33 stimulation in patients with moderate to severe atopic dermatitis 57 days after etokimab injection [84]. In another open-label trial in patients with peripheral spondyloarthritis treated with secukinumab, an anti-IL17A antibody, T cell-engaging SEB *ex vivo* stimulation of whole blood showed efficient inhibition of IL-17A release, but no interference with the induction of central host defense cytokines [85].

In general, previous use of whole blood stimulation assays was mainly restricted to readout pathways known to be affected by the drug under study. While this targeted approach is very helpful in defining target engagement and bioactivity of the drug, the assessment of a broader spectrum of cellular parameters or even genome-wide assessments would simultaneously unravel potential side effects and thereby could help to better stratify patients allowing exclusion of patients with elevated risk profiles.

Single-cell RNA-sequencing as a readout of whole blood stimulations

In the last years, single-cell RNA-sequencing (scRNA-seq) has risen as an important tool to study immunological questions, starting from characterization of cell types at steady state to investigating disease-related changes in complex clinical cohorts [86].

Single-cell transcriptomics provides a comprehensive approach for profiling the whole transcriptome of single cells in a high throughput manner. Although these methods provide high-dimensional, sensitive readouts capturing important biological insights, they are also easily affected by sample handling and require significant attention concerning experimental details, highly sophisticated compute infrastructures, and profound expertise in data analysis [86]. Application of single-cell transcriptomics to clinical scenarios bears further challenges, including logistics, communication within highly interdisciplinary teams, and elaborated clinical and experimental documentation and metadata organization. When highly fragile cell types are part of disease pathology, as can be the case for granulocytes in blood, patient samples have to be processed immediately because such cells do not survive cryopreservation.

Further, steady state analysis of fresh blood closely preserves cell states as physiologically present and therefore allows to study cell types and alterations in specific subtypes without the requirement for isolation and purification of cell populations beforehand. These aspects make scRNA-seq a powerful tool for exploratory studies and, in the context of *ex vivo* stimulations, enables the identification of the cells directly responding to the stimulus as well as indirect responses, for example, due to cell-to-cell communication.

Several studies have utilized scRNA-seq as readout for *ex vivo* stimulation of human blood immune cells, but are mostly applying stimulation to isolated cell types (e.g. [87, 88]) or purified PBMC (e.g. [89, 90]). In a pilot study, the combination of whole blood stimulation with single-cell transcriptomics as a cellular readout has been reported for patients with juvenile idiopathic arthritis [91]. Single-cell transcriptomic analysis identified monocytes as the strongest responding cell type to TNF stimulation, as expected, though granulocytes had been excluded due to PBMC isolation after stimulation. Disease-related transcriptional profiles were not yet discovered, which was most likely due to low sample size and interhuman variability. While not yet fully illustrating the expected power of combining whole blood cell stimulation and single-cell transcriptomics, this example indicates the clinical potential.

Conclusion and future perspectives

Whole blood stimulation assays combined with high-dimensional data acquisition provide a unique opportunity to contribute to a better understanding of human immunity and disease trajectories beyond existing immunological approaches. They have the potential to change diagnostic procedures, optimize treatments, and accelerate or enhance drug development pipelines. Further miniaturization, automation, standardization, and thus heavy cost reduction of current assay systems will be a prerequisite for these methods to be widely used both in the research setting as well as routine clinical applications and this will pave the way to determine the data layers and number of necessary parameters (e.g. number of genes or proteins) that will help improve clinical diagnoses, therapy choices, and/or monitoring. These systems may have the potential to become the working horse for

human immunology and we envision to map individual profiles in the future. As an interim phase, such an approach may be implemented in a discovery phase alongside clinical trials while the biomarkers derived from high-dimensional data may then be translated into more clinically applicable procedures. They may also form the basis of a new disease ontology defining cellular and molecular endotypes of pathologies as a prerequisite for patient stratification, at least for those diseases with a clear involvement of the circulating immune cell compartment. Altogether, we are convinced that the assessment of blood immune cell activation will become an essential part of future precision medicine approaches.

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Abbreviations: **CyTOF:** cytometry by time-of-flight · **PBMCs:** peripheral blood mononuclear cells

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