PRECISION MEDICINE IN CHRONIC INFLAMMATION

EDITED BY: Stefan Schreiber, Oliver Distler, Stefan Niemann, Gabriela Riemekasten and Ralf J. Ludwig

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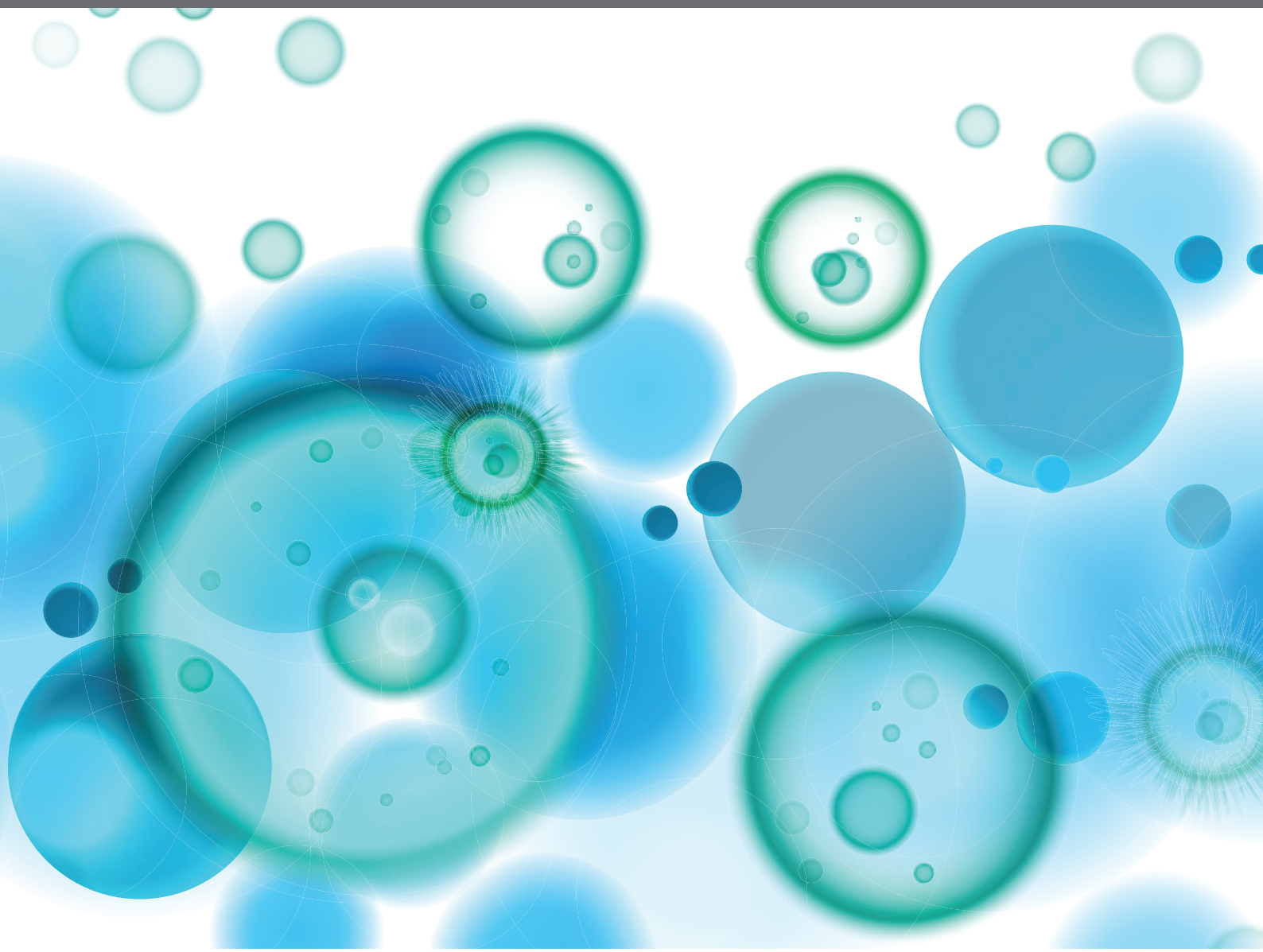

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PRECISION MEDICINE IN CHRONIC INFLAMMATION

Topic Editors:
Stefan Schreiber, CAU Kiel
Oliver Distler, University of Zurich, Switzerland
Stefan Niemann, Research Center Borstel (LG), Germany
Gabriela Riemekasten, University of Lübeck, Germany
Ralf J. Ludwig, University of Lübeck, Germany

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Editorial: Precision Medicine in Chronic Inflammation

Oliver Distler[1], *Ralf J. Ludwig*[2*], *Stefan Niemann*[3,4], *Gabriela Riemekasten*[5] *and Stefan Schreiber*[6]

[1] Department of Rheumatology, University Hospital Zurich, University of Zurich, Zurich, Switzerland, [2] Lübeck Institute of Experimental Dermatology and Center for Research on Inflammation of the Skin, University of Lübeck, Zurich, Germany, [3] Research Center Borstel, Molecular and Experimental Mycobacteriology, Borstel, Germany, [4] German Center for Infection Research (DZIF), Partner Site Hamburg-Lübeck-Borstel-Riems, Borstel, Germany, [5] Department of Rheumatology, University of Lübeck, Lübeck, Germany, [6] Department of Medicine I, University Hospital Schleswig-Holstein, Kiel, Germany

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Editorial on the Research Topic

Precision Medicine in Chronic Inflammation

The principle of precision medicine is most established in oncology; e.g. choice of particular treatment based on the presence of certain molecular alterations within the tumors (1). This implementation of precision medicine has significantly improved the prognosis across many malignant diseases (2). Compared to oncology, precision medicine is still in its infancy in chronic inflammatory diseases – exemplified for pemphigus and pemphigoid diseases (Bieber et al.) herein. However, implementation of precision medicine for chronic inflammatory diseases, such as chronic infectious diseases, inflammatory bowel disease, inflammatory rheumatic diseases and chronic inflammatory diseases of the skin, is expected to have a significant impact of patient well-being (3). There are three key pillars of precision medicine that will enable its implementation into clinical use: (i) identification of unique disease-associated characteristics in individual patients (ii) personalized experimental models of chronic inflammation, and (iii) implementation of personalized treatments. All of these are highlighted in the articles of the Research Topic *Precision Medicine in Chronic Inflammation*, and are shortly introduced in this Editorial.

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Pietro Ghezzi,
Brighton and Sussex Medical School, United Kingdom

***Correspondence:**
Ralf J. Ludwig
ralf.ludwig@uksh.de

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IDENTIFICATION OF UNIQUE DISEASE-ASSOCIATED CHARACTERISTICS IN INDIVIDUAL PATIENTS

At the forefront of precision medicine is the ability to identify unique characteristics in individual patients allowing to select a tailored treatment. This requires techniques that go well beyond currently implemented diagnostic algorithms. Among methods that allow to differentiate between individual patients are (i) assessment of patient-reported outcomes, (ii) *in vivo* imaging up to the cellular level, (iii) detailed phenotyping of the (mal)adaptive immune responses, (iv) molecular characterization allowing to generate polygenic risk scores, which, in case of infectious diseases, also encompasses the pathogens' genetics, as well as (v) drug monitoring.

In this Research Topic, Tran et al. review the currently available patient-reported outcomes (PRO) for chronic inflammatory diseases, their limitations and challenges of addressing these. Especially the longitudinal use of PROs is well-suited to monitor the patient's perception on the quality of life,

disease activity, functional capacity, as well as psychological health. In turn, this enables to capture response (or lack thereof) to treatments in a much more comprehensive and individualized manner (4). Thus, PROs are not only important for clinical management, but also are a cornerstone of more individualized therapies because they directly take individual PROs into account. However, currently clinically implemented PROs only partially reflect actual disease activity. Thus, further development and broader implementation into daily clinical care, of PROs capturing disease activity more precise for individualized therapy guidance is essential for personalized medicine.

Immunophenotyping (Humrich et al.) at a molecular and cellular resolution is a key for classification of patients with chronic inflammatory diseases as it allows to unravel disease pathogenesis and potentially allows to define diagnostic and/or prognostic biomarkers for patient stratification and personalized treatments. Application of unbiased multi-OMICS techniques, such as antigen-specific T cell enrichment (5, 6) or multiplex determination of the activity of 150 kinases (PamGene) (7) are examples of these techniques. The bottleneck of a broader implementation of immunophenotyping are the relative high costs and the integration in a biological and clinical context. For the latter, a close cooperation among clinicians, scientists and systems biologists is essential. Thus, as the tools for phenotyping are available, it will only be a matter of time to explore the full potential of immunophenotyping in inflammation medicine. An example for a biomarker for disease-progression is the presence of myeloid-derived suppressor cells (MDSCs) in hemodialysis patients (Xing et al.): in this patient cohort, persistent high levels of M-MDSCs were associated with higher incidence of stock, heart failure and death. Interestingly, compared to plasma from healthy controls, plasma of hemodialysis patients induced M-MDSCs. This induction of M-MDSCs was sensitive to IL-6 blockade, which may represent a future therapeutic approach for these patients (8). Another example for emerging biomarkers are regulatory autoantibodies to G protein-coupled receptors (GPCR) (Riemekasten et al.), which may reflect the GPCR signature and therefore, the interplay between individual external factors (e.g. microbiome, toxic agents) and internal factors such as the genetic predisposition. Increased or decreased serum concentrations of these autoantibodies lead to clinical disease manifestation (9) and serve as biomarkers for disease progression (10).

Hübenthal et al. highlight the current developments in clinical sequencing and discuss the clinical applicability of polygenic risk scores with regard to chronic inflammatory diseases, such as atopic dermatitis (AD), inflammatory bowel disease (IBD) and coronary artery disease (CAD). Sequencing-based high throughput methods allowed for a (relative) cost-effective sequencing of large patient cohorts. This has and continues to improve our understanding of the genetic background of disease pathogenesis (11–13). In perspective, this data will be the basis for biomedical innovation that potentially allows to for patient stratification at a more individualized level.

Imaging inflammation up to the cellular level is another important pillar for precision medicine. In their overview, Medina et al. review the recent development in imaging of inflammation. Regarding cellular imaging, two-photon microscopy (TPM) for sectioning-free virtual hematoxylin and eosin (H&E) "staining" and optical coherence tomography (OCT) for visualization of cutaneous inflammation. For TPM tissue samples stained with acridine orange (nuclei) and sulforhodamine 101 (counterstain) that leads to an H&E compatible staining. Imaging of unsectioned tissue specimen is then performed using TPM. Ultimately, a digital H&E-equivalent image is generated ready for histological assessment is created from the acquired data. Pending further validation, this workflow of virtual H&E imaging using TPM may represent a faster alternative to conventional histology in the future. The potential of OCT imaging for cellular *in vivo* imaging of the skin has been demonstrated (14). Thus, analysis of cellular morphology combined with dynamic processes of immune cells potentially allows a marker-free "optical biopsy" of skin inflammation using OCT.

Especially in chronic infectious inflammatory diseases, for example tuberculosis, personalized medicine is more and more implemented into clinical care. Biomarker-based treatment decisions, therapeutic drug monitoring and tailored treatments based on the pathogens' genome are presented in-depth in the review articles by Lange et al. and by Merker et al.

Overall, availability and implementation of those measures will allow individualized treatment decisions. We expect implementation of these in the near future, as respective clinical trials are currently performed. For example, a biomarker discovery trial in prospective cohorts from patients with chronic inflammatory diseases for the definition of disease control, headed by Dr. Schreiber, Dr. Thaci and Dr. Weidinger, or a clinical trial on individualized antibiotic therapy for chronic lung infections headed by Dr. Lange, Dr. Rabe, Dr. Niemann and Dr. Schulenburg, all affiliated with the Cluster of Excellence *Precision Medicine in Chronic Inflammation*.

PERSONALIZED MODEL SYSTEMS

Current pre-clinical model systems usually rely on inbred rodents because of the lower variability of the obtained results (15). Yet, diversity in model systems is a key pre-requisite for basic and translational research in precision medicine. Herein, Tran et al. review the potential use of stem cells and organoid technology in precision medicine in inflammation and highlight the use of organoids from human tissues (16, 17).

EMERGENCE OF PERSONALIZED TREATMENTS

Diet is well recognized as an important factor in the pathogenesis of chronic inflammatory diseases, as well as treatment responses (18, 19). Despite this understanding, work demonstrating therapeutic activity of personalized nutrition in chronic

inflammation remains scarce. Yet, based on findings in experimental models (20, 21), implementation of precision nutrition in chronic inflammation (Demetrowitsch et al.) will potentially become a therapeutic and/or preventive measure. In line with this notion, insulin is a key factor in host defense as demonstrated by Casagrande et al. Comorbidity of chronic inflammatory diseases is already used for treatment decisions, which is a step towards personalized medicine. For example, use of TNF inhibitors (Zamri and de Vries) in rheumatoid arthritis patients improved periodontal health. As shown by Sang et al., another possibility to personalize medicine could be a targeted delivery of immunomodulatory drugs based on the cell types present in inflammation.

PERSPECTIVES

Identification of unique disease signatures in individual patients is the key to precision medicine in chronic inflammation. This also encompasses the use of personalized model systems for better understanding of disease pathogenesis and selection of treatments. Ultimately, this will lead to the implementation of personalized treatments for patients affected by chronic inflammatory diseases. The future will see that the phenotypes of inflammatory diseases will disintegrate into many rare diseases with targeted therapeutic approaches in small segments of patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Evolutionary Approaches to Combat Antibiotic Resistance: Opportunities and Challenges for Precision Medicine

Matthias Merker[1,2,3]*, *Leif Tueffers*[3,4], *Marie Vallier*[3,5], *Espen E. Groth*[3,4,6], *Lindsay Sonnenkalb*[1], *Daniel Unterweger*[3,5], *John F. Baines*[3,5], *Stefan Niemann*[1,2,3†] and *Hinrich Schulenburg*[3,4†]

[1] Molecular and Experimental Mycobacteriology, Research Center Borstel, Borstel, Germany, [2] German Center for Infection Research (DZIF), Partner Site Borstel-Hamburg-Lübeck-Riems, Hamburg, Germany, [3] Cluster of Excellence Precision Medicine in Chronic Inflammation, Kiel, Germany, [4] Evolutionary Ecology and Genetics, Zoological Institute, Christian-Albrechts-Universität, Kiel, Germany, [5] Section of Evolutionary Medicine, Institute for Experimental Medicine, Kiel University and Max Planck Institute for Evolutionary Biology, Plön, Germany, [6] Department of Internal Medicine I, University Hospital Schleswig-Holstein, Kiel, Germany

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Edited by:
Ralf J. Ludwig, University of Lübeck, Germany

Reviewed by:
Antoine Abou Fayad, American University of Beirut, Lebanon
Elsa Anes, University of Lisbon, Portugal

***Correspondence:**
Matthias Merker
mmerker@fz-borstel.de

† These authors have contributed equally to this work

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The rise of antimicrobial resistance (AMR) in bacterial pathogens is acknowledged by the WHO as a major global health crisis. It is estimated that in 2050 annually up to 10 million people will die from infections with drug resistant pathogens if no efficient countermeasures are implemented. Evolution of pathogens lies at the core of this crisis, which enables rapid adaptation to the selective pressures imposed by antimicrobial usage in both medical treatment and agriculture, consequently promoting the spread of resistance genes or alleles in bacterial populations. Approaches developed in the field of Evolutionary Medicine attempt to exploit evolutionary insight into these adaptive processes, with the aim to improve diagnostics and the sustainability of antimicrobial therapy. Here, we review the concept of evolutionary trade-offs in the development of AMR as well as new therapeutic approaches and their impact on host-microbiome-pathogen interactions. We further discuss the possible translation of evolution-informed treatments into clinical practice, considering both the rapid cure of the individual patients and the prevention of AMR.

Keywords: AMR, drug resistance, evolution, precision medicine, evolutionary medicine

INTRODUCTION

The evolution of antimicrobial resistance (AMR) in bacteria by mutation of the chromosome or horizontal gene transfer is a naturally occurring phenomenon that can be observed even in the absence of human interventions (1, 2). However, it is the widespread use of antibiotics for almost a century that led in the actual public health crisis. Indeed, numerous pathogens show an increase in their antimicrobial resistance (AMR) levels, often directed at multiple antibiotic drugs (i.e., multidrug resistance, MDR). Infections caused by MDR pathogens are difficult, if not impossible to treat with the most commonly used drugs, thereby requiring the application of less effective and/or more toxic regimens. By the year 2050, more patients are expected to die from infections with MDR pathogens (10 million/year) than from cancer today (8.2 million/year) (3). Current treatment

practices, exemplified by proverbs such as "One size fits all" and "Hit hard and hit early" at least partially contributed to this scenario, and are unlikely to suffice for the challenges that medicine will face in the future (4).

The field of Evolutionary Medicine strives to combat increasing drug resistance rates by exploiting evolutionary principles of resistance emergence and spread to design new treatment concepts (5, 6). The aims are 3-fold: (i) reducing intra-patient resistance selection that can lead to treatment failure, (ii) providing more rapid and less toxic cures to individual patients, and (iii) reducing the likelihood of AMR evolution and transmission at the population level. Achieving these aims is particularly challenging, because individualized patient care and public health considerations do not necessarily align. In particular, treatment regimens that were conventionally optimized to eliminate an infection as fast as possible impose a strong, often monotonic selective pressure on causal pathogens. Depending on bacterial adaptive capabilities, this can potentiate the risk for both short- and long-term resistance development (7). Furthermore, a broad activity of antimicrobials, which is often required for effective empirical anti-infective treatment, can compromise the healthy microbiome and thereby bears a risk for the development or aggravation of secondary diseases (8). Thus, we need to carefully consider evolutionary trajectories of pathogens in response to drug therapy, host immune system, as well as within a complex ecosystem of commensal microbial communities (**Figure 1**). In this context, Evolutionary Medicine is inevitably connected to applied Precision Medicine, where patients are stratified based on host, microbiome and pathogen characteristics, in order to deliver the most effective and sustainable treatment for a particular patient or group (9).

Here, we review (1) principles of bacterial resistance evolution and currently proposed concepts of evolution-informed therapies, (2) related challenges and opportunities, particularly with regard to host-microbiome-pathogen interactions, and (3) their relevance for clinical practice and Precision Medicine in the future.

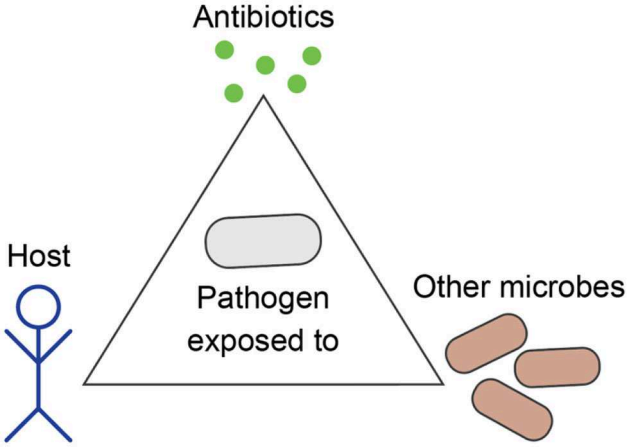

FIGURE 1 | Evolution of bacterial pathogens during infection is influenced by the host's characteristics, other microbes, and antibiotics.

PRINCIPLES OF DRUG RESISTANCE AND EVOLUTION INFORMED THERAPIES

Drug Resistance Mechanisms and Possible Fitness Costs

Throughout the course of evolution, molecules with antimicrobial activity were naturally selected in different organisms in order to outcompete bacteria in their environment by targeting essential functions such as DNA replication, transcription, translation, or metabolism (10). Although drug resistance evolution represents a paramount example of evolution observable in real-time, the design of antibiotic treatment regimens most often ignores the obtained insights into the involved selective processes (11). In fact, resistance evolving under antibiotic treatment in humans was traditionally seen as a phenomenon in individual patients (12, 13). Even if resistance-mediating adaptations arise in this context, their overall effect is assumed to be detrimental and changes may lead to lower fitness, so that resistant strains would usually not outcompete their susceptible counterparts (13). Thus, treatment designs over the past decades mostly focused on the principle of "Hit hard and hit early," which goes back to the pioneering work of Paul Ehrlich from over 100 years ago ("*frapper fort et frapper vite*") (14). Interestingly, Ehrlich already recognized the "great power of adaptation" and the risk for relapse and resistance evolution if a small number of pathogenic bacteria survives treatment (15). In fact, within the last decades, several pathogens acquired MDR, which prevailed under constant antibiotic selective pressure in hospitals and maintained transmissibility in the population (9).

One explanation for the success of AMR pathogens is the selection of low-cost or even no-cost resistance mediating mutations as shown for pathogens of the *Mycobacterium tuberculosis complex* (Mtbc), the causative agent of tuberculosis. The most prevalent mutations that mediate MDR, i.e., resistance against the widely used anti-tuberculous drugs isoniazid (katG p.S315T) and rifampicin (rpoB p.S450L), confer almost no fitness cost compared to wildtype strains (16, 17). Thus, even in the absence of the selective pressure of antibiotic therapy, MDR Mtbc strains persist and are associated with MDR tuberculosis outbreaks mainly in Eastern Europe, Central Asia, and South Africa (18, 19). Some resistance mechanisms, especially efflux pumps, are under extensive transcriptional control and can be amplified while antibiotics are present, so that any associated fitness costs are removed once resistance can be scaled back (20–22). Unlike Mtbc strains that evolve purely clonally, other bacteria such as *Pseudomonas aeruginosa*, an opportunistic pathogen in chronic lung infections, can acquire drug resistance not only by mutation, but also via horizontal gene transfer (HGT), e.g., involving plasmids (23). Such mobile resistance elements allow for the loss of AMR-mediating genes in the absence of antibiotic exposure, possibly attenuating AMR-associated fitness costs (24).

Another important evolutionary phenomenon observed in a number of AMR bacteria is compensatory evolution.

Compensatory mutations are secondary mutations that reduce or ameliorate the fitness costs by the resistance mutation itself (25, 26) and have been described in a number of bacterial species *in vitro* (27, 28), and *in vivo* (28, 29), and have been associated with enhanced transmissibility (18, 19). Compensatory mutations can be clinically relevant, as they maintain resistance alleles and/or resistant clones in bacterial populations.

Beyond genetic mechanisms that allow bacteria to resist antibiotics or compensate for associated fitness costs, several species are able to express specific traits that protect them against antibiotics. *P. aeruginosa*, for example, forms biofilms and drug tolerant persister cells, leading to phenotypic resistance, often associated with chronic infection and relapse in patients with cystic fibrosis (30). *M. tuberculosis* has a unique thick cell wall and evolved a life cycle that mostly takes place in a granuloma (i.e., a concise structure of immune cells), which maintains the pathogen locally at the site of infection (e.g., the lung tissue) and shields from antibiotic penetration (31). Mtbc strains can also shift to a dormant state that reduces metabolic activity and increases the resistance to host/environmental stresses, including antibiotics (32).

These examples illustrate that drug resistance is a multifaceted evolutionary process, and that different pathogens have evolved diverse strategies which cause the traditional assumptions of antimicrobial therapy to fail. An in-depth understanding of these mechanisms may help to refine anti-infective therapies with currently available drugs, for example by focusing on antimicrobials, for which resistance comes at high costs (9).

From a public health perspective, resistance, and related bacterial fitness have to be extended to a broader context in which the (environmental) transmissibility of human pathogens and man-made selective pressures due to population-level antibiotic usage and its environmental distribution are considered (33). Moreover, the optimization of treatment strategies always has to be aligned with public health programs, in order to take account of insufficient disease control and surveillance measures as well as poor pharmaceutical control (e.g., availability of antibiotics in supermarkets), as observed in some structurally developing regions (34).

Synergistic and Antagonistic Combination Treatments

MDR infections are often treated with drug combinations. Such combination therapy follows the rationale that the simultaneous emergence of resistance against employed drugs is less likely than that against single drugs. In detail, resistance against a hypothetical drug A (conferred by a mutation) may arise rapidly by chance, depending on the mutation frequency and the population size of the infecting pathogen. In contrast, the combined probability of the simultaneous occurrence of mutations conferring resistance against two different drugs A and B in any one cell is very low, not considering cross-resistance.

Combination therapy is often designed to enhance treatment efficacy by combining drugs that mutually enhance each other. A hypothetical drug pair A and B usually interact additively. Each drug individually may inhibit bacterial growth by 50% at a given concentration, in combination a theoretical growth reduction of 75% (residual growth $= 0.5 \times 0.5$) is expected. The effect on growth can further be influenced by physiological interactions, so that antibiotic combinations become antagonistic (combination causes less growth reduction than expected based on the additive effect) or synergistic (combination leads to a higher growth reduction than expected) (35). Synergistic drug combinations that mutually improve their therapeutic effects should enhance treatment efficacy. Counterintuitively, however, several studies suggested that in the long-term they may rather promote the spread of resistance, possibly as a consequence of competitive release (i.e., the stronger effect eliminates competitors so that the surviving resistant variants obtain enhanced access to resources) (36–39).

Combination treatment may also select for mutations that confer resistance to both drugs simultaneously, instead of a sequence of individual drug resistances. De-repression of efflux pump expression is one example (40). While efflux pumps are generally upregulated under cellular stress, leading to temporary drug resistance, this upregulation can be made permanent through loss-of-function variants of repressor genes, as repeatedly observed in clinical isolates (41).

A recent systematic analysis of combination therapy in *P. aeruginosa* suggests that the picture is more complex. A comprehensive set of more than 1,600 evolution experiments and 38 distinct combination treatments demonstrates that synergistic antibiotic combinations increase the extinction of bacterial populations, even if drugs are used at sub-lethal level, while no clear effect on adaptation rates was observed in the surviving populations (42). Instead, evolutionary trade-offs appear to be the prime determinant of combination efficacy, in particular when these trade-offs take the form of evolved collateral sensitivities (see below), leading to significant reductions in adaptation rates, at least under the experimental conditions (42).

Sequential Treatments

Sequential antibiotic treatment refers to the administration of different drugs in a chronological sequence to an individual patient, rather than their simultaneous combination, and in distinction to hospital drug rotation protocols (6).

Fundamentally, antibiotic monotherapy and sequential treatment both expose the patient and the infectious agent to one drug at a time. Evolutionary theory and *in vitro* experiments predict that bacteria are often able to adapt easily to a singular, continuous selective pressure (6, 43, 44). Consequently, *in vivo* observations confirm that resistance evolution indeed can happen within few days within patients (45). Antibiotic resistance phenotypes may be present at low frequencies in the infecting population or arise through spontaneous mutation, and subsequent selection by the antibiotic pressure (46). In a sequential treatment, however, selection of resistant subpopulations is repeatedly interrupted by changing the selective pressure. This strategy alone is able to slow the evolution of resistance down (47), and can be further amplified by limiting bacterial resistance evolution itself, through collateral sensitivity and negative hysteresis.

Collateral sensitivity (CS) was first described in the early antibiotic era (48), and has recently resurfaced as a promising strategy to limit bacterial resistance evolution (49). The effect results from an evolutionary trade-off, in which a mutation conferring resistance to one drug simultaneously causes increased sensitivity to another drug. The phenomenon of CS has now been described for a variety of bacterial taxa, such as *Acinetobacter baumannii*, *Eschericia coli*, *Enterococcus faecalis*, *P. aeruginosa*, or *Staphylococcus aureus*. It's strength and prevalence might depend however on the drug sequence, the bacterial species, and the evolutionary lineage within a bacterial species (44, 49–56). Overall, CS shows high potential for precision medicine to tailor treatments specific to a pathogen or even a specific strain (49, 57).

Whilst CS is based on changes in molecular structure (genetic mutations) occurring during resistance evolution, the concept of negative hysteresis (NH) results from a physiological response. In particular, NH occurs when the application of one antibiotic increases the efficacy of a second, subsequently applied antibiotic (58). Thus, increased susceptibility is not based on mutation and selection, but caused by physiological changes within a bacterial generation, even at sub-lethal antibiotic concentrations. NH may enhance the efficacy of sequential treatments, even at locations of poor drug penetration (e.g., biofilms) or with elevated levels of resistance (58).

HOST-MICROBIOME-PATHOGEN INTERACTION

In medical practice and research, pathogens are often studied as stand-alone entities that grow independently from other microbes and evolve antibiotic resistance as an adaptation to anti-infective therapy. However, pathogens are part of a complex ecosystem within the host, which can greatly influence the outcome of anti-infective treatment and the occurrence of AMR. During infection, pathogens evolve not only to resist antibiotics, but also to increase their fitness within the particular host in competition to the commensal microbiota, which needs to be taken into account when applying evolutionary approaches to medicine (**Figure 1**).

The spectrum of colonizing bacteria can be influenced by a variety of host mechanisms. For example, gastrointestinal glycans such as those generated by the enzyme encoded by the blood-group related gene *B4galnt2* were shown to be involved in shaping the resident microbiota (59) and, importantly, play a role in susceptibility to infection, e.g., by *Salmonella* (60).

Moreover, bacteria are forced to interact with other microbes upon colonization or infection of the host. In the process, commensal microbes impact the eco-evolutionary dynamics by affecting traits under selection (61). For example, mono-colonization of the mouse gut by *E. coli* can lead to predictable bacterial metabolic adaptations through the selection of mutations in genes related to amino acid metabolism, a process that is, however, altered by co-colonization with other microbes (61). Another example is colonization resistance, where presence of a complex residing microbial community can protect against pathogen infections (62).

Importantly, host-microbe and microbe-microbe interactions translate to ecological and evolutionary pressures that exhibit clinical relevance. These eco-evolutionary mechanisms affect both symbiotic organisms and pathogens, making their understanding key to sustainable pathogen control.

Furthermore, several known forms of bacterial interaction are directly linked to antimicrobial resistance.

Bacterial Co-operation

During infection, pathogens can engage in cooperative behavior. For example, *P. aeruginosa* in the lungs of cystic fibrosis patients acquires iron via the siderophore pyoverdin, which is costly to produce. Pyoverdin is secreted into the bacterial environment where it represents a public good: Either it gets taken back up in its iron-loaded form by the secreting cell, thereby offsetting the cost of its production, or by a non-pyoverdin-producing bacterium, which thereby benefits from iron uptake without paying the cost of siderophore production (63–65). Resistance to β-lactam antibiotics can be mediated in a similar cooperative manner: secreted β-lactamases act as a public good by simultaneously protecting bacteria that do not produce the enzyme (66–68).

Bacterial Competition

Bacteria-bacteria killing is a form of bacterial competition and can be mediated by the type VI secretion system (T6SS). In patients with *A. baumanii* infection, however, some bacterial isolates display an inactivated bacterial killing machinery whilst carrying several antimicrobial resistance genes (69). In these isolates, a large, self-transmissible plasmid encoded genes for resistance toward antibiotics ranging from beta-lactams to chloramphenicol, whilst carrying a negative regulator of T6SS. Only bacteria that could suppress their T6SS (thereby reducing elimination of surrounding bacteria) were able to successfully transmit the AMR-encoding plasmid containing the T6SS suppressor to other microbes, additionally facilitating the transmission of other AMR-encoding plasmids (70). Thus, a mechanistic linkage to bacteria-bacteria killing mechanisms can directly affect the spread of AMR.

Bacterial Communities

Within an infected host, individual bacteria usually grow as part of communities with specific composition and spatial organization. In the example of *P. aeruginosa* in cystic fibrosis lungs, isolates from one particular site of a patient's lung can show resistance to a particular antibiotic, whereas bacteria from other sites of the same lung do not (71). Laboratory studies supported by computational modeling previously detected different levels of AMR depending on the density of the community and the levels of bacterial mixing (72, 73). As another example, *E. coli* may either tolerate higher concentrations of antibiotics (74) or, alternatively, be prevented to evolve resistance when embedded in a diverse bacterial community, highlighting the importance of bacterial communities for AMR of pathogens (75).

In a nutshell, bacterial pathogens are part of complex ecological environments, where their evolutionary dynamics are heavily affected by host-microbe and bacteria-bacteria interactions with cooperation, competition and community organization directly affecting resistance to antimicrobials. Looking ahead, the challenge will be to incorporate these aspects of a host's ecology and evolutionary history, and bacterial life into our understanding of AMR to inform therapy design.

RELEVANCE FOR CLINICAL PRACTICE AND PRECISION MEDICINE

Currently favored policies to counter AMR foremost focus on limiting the use of antibiotics to necessary medical treatments and encouraging the development of new antimicrobial substances (76). Whilst these measures are self-evident, they are not sufficient to provide a sustainable solution to the problem on their own. Evolutionary adaptation is at the very core of the bacterial AMR crisis. Therefore, the development of future treatment strategies against bacterial infections should necessarily follow evolutionary principles. Newly developed antimicrobial substances will at most provide a temporary relief, as evolution is a continuously ongoing process and almost certainly, resistance will eventually emerge against any new substance. Approaches following the "hit hard and hit early" principle, present since the beginning of the antibiotic era, need to be refined, taking into account individual characteristics of the infecting pathogen lineages, the affected patient, and the associated microbiota. Especially chronic recurring infections (such as *P. aeruginosa* in cystic fibrosis) or long-term antibiotic use (such as tuberculosis therapy) highlight that in numerous cases, due consideration should be given to avoid promoting resistance unintentionally by merely focusing on the simplicity of therapy. Even though evolution informed treatment strategies such as those outlined above might come with more complicated and time intensive protocols than those currently applied, they have the likely advantage to reduce the rate of resistance evolution and thereby improve therapy outcome, not only in individual patients, but also on the population level.

The complex interactions of pathogens with the host, local environments and residential microbiota render their evolutionary responses to antimicrobial substances when applied *in vivo* just as complex. This is where Precision Medicine comes into play. There is an urgent need to characterize bacterial infections, including identification of the pathogen taxa, the site of infection(s), phenotypic and genotypic drug resistance profiles, but also individual host and microbiome characteristics for better and more sustainable treatments. Developing and applying tailored treatments, however, comes with challenges inherent to Precision Medicine, requiring thoughtful balancing of individual and public interests: Deep sampling of pathogens in an infection for current explorative research as well as for future diagnostics or therapy surveillance, in conjunction with comprehensive characterization of patients in a system medicine approach comes with a high demand of personal and material resources. If patients are prone to undergo more intensive and invasive procedures than currently required when adhering to established treatment concepts, physicians may run into an ethical dilemma when weighing the benefit for the individual patient against the benefit for the public. However, evolution-informed therapies may offer an opportunity to meet both of these requirements by ensuring pathogen elimination and minimizing resistance spread, thereby sustaining treatment efficacy.

AUTHOR CONTRIBUTIONS

MM, LT, MV, EG, LS, and DU drafted the manuscript. MM, SN, and HS conceived the manuscript. All authors made intellectual contributions, critically revised the manuscript, and gave final approval.

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Perspective for Precision Medicine for Tuberculosis

Christoph Lange[1,2,3,4,5]*, *Rob Aarnoutse*[6], *Dumitru Chesov*[1,2,3,7], *Reinout van Crevel*[6], *Stephen H. Gillespie*[8], *Hans-Peter Grobbel*[1,2,3], *Barbara Kalsdorf*[1,2,3,4], *Irina Kontsevaya*[1,2,3], *Arjan van Laarhoven*[6], *Tomoki Nishiguchi*[9], *Anna Mandalakas*[9], *Matthias Merker*[2,4,10], *Stefan Niemann*[2,4,10], *Niklas Köhler*[1,2,3], *Jan Heyckendorf*[1,2,3], *Maja Reimann*[1,2,3], *Morten Ruhwald*[11], *Patricia Sanchez-Carballo*[1,2,3], *Dominik Schwudke*[2,12,13], *Franziska Waldow*[2,12] *and Andrew R. DiNardo*[9]

[1] Division of Clinical Infectious Diseases, Research Center Borstel, Borstel, Germany, [2] German Center for Infection Research (DZIF) Partner Site Borstel-Hamburg-Lübeck-Riems, Borstel, Germany, [3] Respiratory Medicine and International Health, University of Lübeck, Lübeck, Germany, [4] Cluster of Excellence Precision Medicine in Chronic Inflammation, Kiel, Germany, [5] Department of Medicine, Karolinska Institute, Stockholm, Sweden, [6] Department of Internal Medicine, Radboud Center of Infectious Diseases (RCI), Radboud University Medical Center, Nijmegen, Netherlands, [7] Department of Pulmonology and Allergology, Nicolae Testemitanu University of Medicine and Pharmacy, Chisinau, Moldova, [8] School of Medicine, University of St Andrews, St Andrews, United Kingdom, [9] The Global Tuberculosis Program, Texas Children's Hospital, Immigrant and Global Health, Department of Pediatrics, Baylor College of Medicine, Houston, TX, United States, [10] Molecular and Experimental Mycobacteriology, Research Center Borstel, Borstel, Germany, [11] Foundation of Innovative New Diagnostics (FIND), Geneva, Switzerland, [12] Bioanalytical Chemistry, Priority Area Infection, Research Center Borstel, Leibniz Lung Center, Borstel, Germany, [13] Airway Research Center North, German Center for Lung Research (DZL), Borstel, Germany

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***Correspondence:**
Christoph Lange
clange@fz-borstel.de

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Tuberculosis is a bacterial infectious disease that is mainly transmitted from human to human via infectious aerosols. Currently, tuberculosis is the leading cause of death by an infectious disease world-wide. In the past decade, the number of patients affected by tuberculosis has increased by ∼20 percent and the emergence of drug-resistant strains of *Mycobacterium tuberculosis* challenges the goal of elimination of tuberculosis in the near future. For the last 50 years, management of patients with tuberculosis has followed a standardized management approach. This standardization neglects the variation in human susceptibility to infection, immune response, the pharmacokinetics of drugs, and the individual duration of treatment needed to achieve relapse-free cure. Here we propose a package of precision medicine-guided therapies that has the prospect to drive clinical management decisions, based on both host immunity and *M. tuberculosis* strains genetics. Recently, important scientific discoveries and technological advances have been achieved that provide a perspective for individualized rather than standardized management of patients with tuberculosis. For the individual selection of best medicines and host-directed therapies, personalized drug dosing, and treatment durations, physicians treating patients with tuberculosis will be able to rely on these advances in systems biology and to apply them at the bedside.

Keywords: precision medicine, tuberculosis, tailor-made regimen, mycobacterial genotypes, endotypes

INTRODUCTION

Tuberculosis is the leading cause of death attributed to a single microbial pathogen world-wide (1). The World Health Organization (WHO) estimates, that in the year 2018 globally 10 million people newly developed tuberculosis and 1.5 million people died from this disease (1). Despite the enormous burden of tuberculosis on healthcare systems, especially in developing countries, research on new preventive and diagnostic methods and novel therapies against tuberculosis has gained new momentum only recently.

For the past five decades, patients with tuberculosis have received the same standard therapies without acknowledging differences in human immunity, pharmacokinetics or variations in the pathogenesis or of the causative microbe, *Mycobacterium tuberculosis*. Treatment was empiric or adopted to results of phenotypic drug-susceptibility testing that takes several weeks to months to become available. Patient-tailoring was limited to adjusting dosing to body weight, mostly in pediatric patients (2). We have now entered an exciting era of medicine with major advances in the field of system biology and there is a realistic perspective that patients with tuberculosis will substantially benefit from these developments (3–5).

In the future, tuberculosis patient care may be individualized in at least 4 areas (**Figure 1**): (I) next generation sequencing of microbial *M. tuberculosis* DNA can predict drug susceptibility in the first week of diagnosis (6); (II) host immunity can be detrimentally suppressed and need augmenting or it can be detrimentally exuberant and need to be suppressed (7–9); or other forms of host genetic variability that may be specifically addressed by immune-based interventions (10), (III) individualized drug concentrations that can guide antimicrobial dosing (11), and (IV) biomarkers that predict relapse-free cure and can guide duration of required therapeutics (12) (**Figure 1**). Such stratified therapies are within reach and could soon become available for the clinical management of tuberculosis.

Precision Medicine refers to prevention and treatment strategies that take individual variability into account and predict,

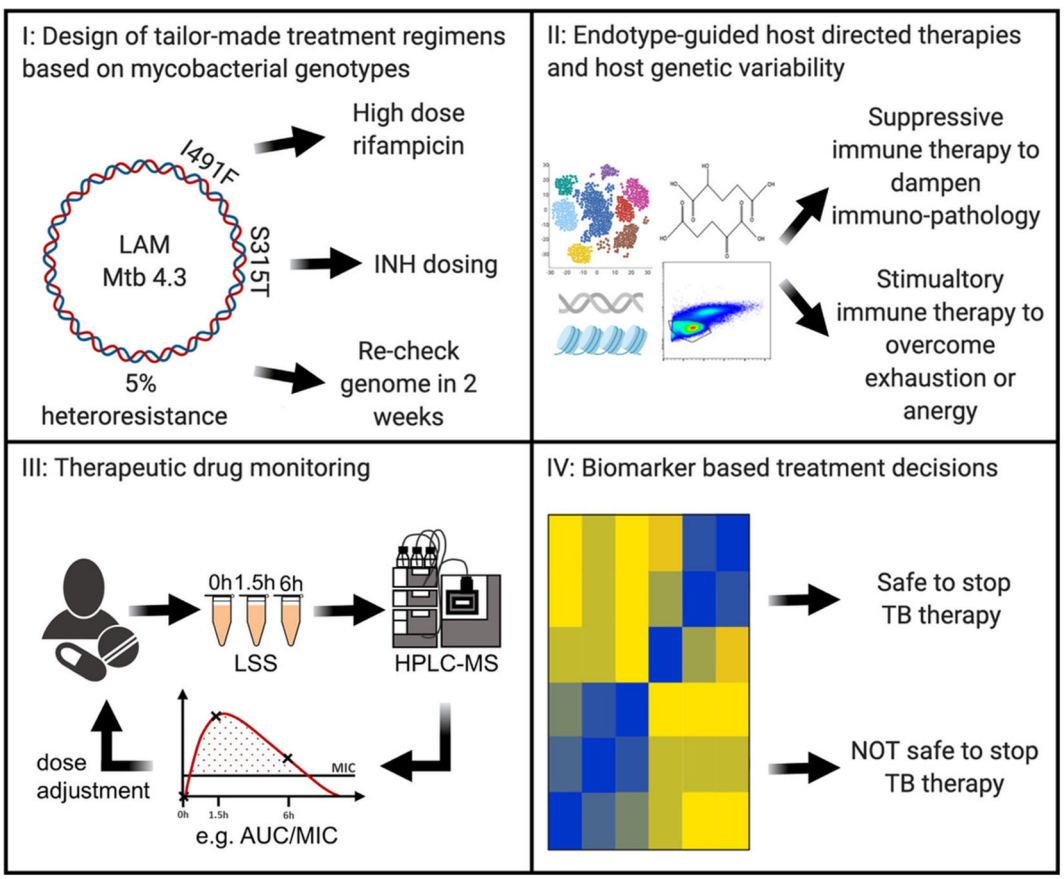

FIGURE 1 | In the near future, Precision Medicine for tuberculosis will likely include (I) antibiotic regimens based on next-generation sequencing of the *Mycobacterium tuberculosis* genome to guide tailor-made therapies; (II) evaluation of gene expression, genetic, epigenetic, metabolism, and/ or immune phenotyping to discern the host endotype with endotype-specific host directed therapies to shorten and improve clinical outcomes; (III) individualization of antibiotic dosing through therapeutic drug monitoring; (IV) in treatment biomarker levels to customize therapy duration. LAM, lipoarabinomannan; Mtb, *Mycobacterium tuberculosis*; INH, isoniazid; LSS, limited sampling strategy; HPLC-MS, high-performance liquid chromatography-mass spectrometry; AUC, area under the curve; MIC, minimal inhibitory concentration; TB, tuberculosis.

who will benefit most from specific therapies and who will not. Taken together, Precision Medicine will improve patient outcome and reduce cost.

SUBSECTIONS RELEVANT FOR THE SUBJECT

I: Design of Tailor-Made Treatment Regimens Based on Mycobacterial Genotypes

Drug resistances in *M. tuberculosis* complex strains are exclusively mediated by genomic variants, mainly single nucleotide polymorphisms (SNPs) and small insertions/deletion (indels). Acquisition of resistance genes via plasmids or horizontal gene transfer does not occur (13). Accordingly, resistant phenotypes of *M. tuberculosis* complex strains mainly have a clear genetic correlate, which means SNPs can be used for resistance predictions with very high precision and are expected to replace phenotypic drug susceptibility testing (DST) in the future (13). The virtually complete interrogation of all resistance associated variants in genomes of clinical *M. tuberculosis* complex strains has become possible by advances of next generation sequencing (NGS) techniques. NGS allows for rapid sequencing of full genomes or targeted sequencing of resistance genes (amplicon sequencing) even directly from patient specimens, i.e., sputum (14–16). While genomic variant detection is of very high accuracy, genotypic drug susceptibility testing performance is strongly dependent on the underlying knowledge database (17). Although there is no standardized and globally adopted, "resistance mutation catalog" available yet, recent studies demonstrate that genotypic DST can predict resistance to the first-line drugs isoniazid, rifampicin, pyrazinamide with more than 90% sensitivity and more than 95% specificity, and thus would meet the WHO target product profile for molecular DST assays (6, 18). However, resistance prediction for some drugs, such as ethambutol and pyrazinamide is problematic due to breakpoint artifacts in phenotypic DSTs (19) or gaps in current knowledge databases, i.e., yet unknown resistance mutations.

There is emerging evidence that genotypic DST has a very good prediction for phenotypic drug susceptibility (20, 21) although for some drugs like PAS, D-cycloserine, thioamides, linezolid, meropenem-clavulanate, bedaquiline, clofazimine, and delamanid data are still limited. Programmes aiming to fill the knowledge gap in "geno-to-pheno" prediction such as CRyPTIC (www.crypticproject.org) and ReSeqTB (platform.reseqtb.org) are currently collecting genome sequencing data of global strain collection in combination with phenotypic DST data to further improve genotypic resistance predictions. Here, sequencing approaches represent an effective alternative to rapidly generate comprehensive resistance profiles of multidrug-resistant (MDR) strains allowing the design of a successful MDR-TB therapy for around 90% of the MDR-TB patients (18, 20).

Genotypic DST methods already provide accurate predictions for first-line anti-tuberculosis drugs and guide the design of MDR-TB therapies (6, 20). Sputum sequencing together with an improved interpretation catalog of drug resistance defining mutations will potentially allow genotypic DST to replace phenotypic DST for a large fraction of clinical *M. tuberculosis* complex strains.

II: Endotype-Guided Host Directed Therapies and Host Genetic Variability

Tuberculosis endotypes are the distinct molecular pathways through which an individual can progress to active tuberculosis. In a rough generalization, tuberculosis host immunity endotypes can be classified as immune deficient or as immune exuberant. The best described tuberculosis endotypes are deficiencies in interleukin (IL)-12, interferon (IFN)-γ or tumor necrosis factor (TNF) pathways, which result in decreased intracellular killing of *M. tuberculosis* (22, 23). However, mouse and human studies also demonstrated that exuberant IFN-γ or TNF is detrimental, resulting in pulmonary and macrophage necrosis and the escape of viable *M. tuberculosis* into extracellular space (8, 24–26). These studies demonstrate that a single immune correlate of protection is unlikely to be identified as the host immunity can not be either deficient or overly exuberant. Therefore, implementation of host-directed therapies (HDT) should preferably not be indiscriminate, but needs to be guided by the tuberculosis immune endotype. Preliminary work supports the identification of divergent host endotypes with contradictory metabolic, epigenetic, and immune gene expression endotypes (7), but these transcriptional studies lack corresponding functional studies.

In the pre-antibiotic era, treatment for tuberculosis was limited to host-directed therapy aiming at improving the nutritional and immune status of patients, e.g., with cod liver oil and sunlight (27), resulting in ∼20% survival rate for a period of at least 10 years (28). Harnessing and targeting host immunity, while minimizing immunopathology is the critical next phase in improving anti-tuberculosis therapy. Implementing endotype-specific host-directed therapy, first require proper characterization of the distinct tuberculosis endotypes in order to identify amendable targets. Tuberculosis dampens host immune responsiveness (29–33), and therefore transcriptomic and immune functional studies must be implemented both at baseline and upon antigenic stimulation. Since 1927, we have known that metabolism drives immunity (34) and modern studies are defining the metabolic-epigenetic-immune axis (35–39). Therefore, ideally, transcriptomic, metabolic, epigenetic and functional immune studies would be paired with robust clinical information that would allow for multi-modal data integration between molecular data and clinical outcomes. Finally, an immune deficient vs. immune exuberant model, while helpful, is overly simplistic as single cell studies have demonstrated deficiency in one arm of immunity, while exuberance in another. For example, tuberculosis induced hemophagocytic lymphohistiocytosis (HLH) is characterized by exuberant monocytes and deficient cytotoxic lymphocytes (40–43).

Endotype characterization studies likely will need to occur at disease diagnosis and again weeks into therapy. Tuberculosis is the archetypical chronic infection, hence host immunity after months of chronic antigenic stimulation is unlikely to

resemble host immunity at the beginning of infection. Upon initial antigenic stimulation, modulated by inositol triphosphate receptors, there is an influx of calcium that helps stimulate glycolysis and activates nuclear factor of activated T-cells (NFAT) to translocate into the nucleus (44) as an example. When immune activation is chronic, NFAT switches from heterodimerizing with activator protein (AP)-1, to forming homodimers that drive anergy and thymocyte selection-associated high mobility group box protein TOX (TOX) and nuclear receptor 4A (NR4A) and epigenetic-mediated immune exhaustion (45–50). Therefore, drugs that modulate calcineurin-NFAT, such as cyclosporin A or tacrolimus, could detrimentally inhibit host immunity early in the disease process by blocking AP-1-NFAT heterodimers that drive beneficial immunity. In contrast, late in the disease process, these same drugs may be beneficial by blocking the detrimental NFAT homodimers that drive anergic and immune exhaustion responses. Evaluating endotypes could be operationalized by tests such as a mycobacterial growth inhibition assay, a test that has identified ∼50% of tuberculosis patients to have good mycobacterial killing capacity and ∼50% to have deficient killing capacity (51), and an ELISA based test, such as a modified version of the QuantiFERON-TB Gold in-tube assay or T-SPOT.TB assay.

Host genetic studies have thus far largely focused on susceptibility to active tuberculosis (52, 53), to a lesser extent on susceptibility to infection (54) while the relationship between host genetic variability and treatment outcome is largely unexplored.

With regard to anti-tuberculous treatment, genetic studies have focused on drug exposure and toxicity ("pharmacogenetics"). The strongest association established is between N-acetyltransferase (NAT)2-acetylator status and isoniazid drug concentrations, microbiological failure, relapse (55), and hepatotoxicity (56) and probably, genetic variation underlies rifampicin bioavailability and aminoglycoside-induced hearing loss (10). A next step would be "Mendelian randomization" studies: associating genetic loci predicting drug concentrations to treatment outcome (rather than toxicity). Then, pharmacogenetic trials could be initiated in which patients are randomized to standard treatment vs. genotype-based drug dosing.

In the most extreme disease phenotypes, targeted HDT may be most relevant. A Leukotriene A4 hydrolase (LTA4H) promotor genotype, important for the balance between pro- and anti-inflammatory eicosanoids (24), predicted cerebrospinal fluid leukocyte count. The hyper-inflammatory genotype was associated with improved survival in HIV-negative patients receiving corticosteroids (57, 58). In contrast, the same LTA4H genotype did not predict mortality in another cohort of patients (59), possibly because of higher disease severity, but the conflicting results could also be driven by genetic factors such as shorter regions of linkage disequilibrium or plasticity in the direction of the effect of the polymorphism (60). This shows that host genetic studies should be replicated in different settings. This can result in randomized controlled trials with stratification according to genotype, as is currently done in a study among patients with tuberculous meningitis, where only those with the hypo-inflammatory LTA4H genotype are randomized to receive corticosteroids or placebo (and all with a hyperinflammatory genotype receive corticosteroids) (61).

Genetic studies can be strengthened by an integrated or "cross-omics" approach. Among patients with tuberculous meningitis, using unbiased metabolomics, it was found that higher cerebrospinal fluid tryptophan, which can affect *M. tuberculosis* growth and central nervous system inflammation, was associated with higher mortality. Using a genome-wide association approach, so-called quantitative trait loci (QTLs) that associate with cerebrospinal fluid tryptophan levels were identified, and those same QTLs predicted survival in 285 other patients (62). The relevance of tryptophan metabolism, its genetic regulation, and the possible implications HDT are now being examined in more than 2,000 tuberculous meningitis patients (https://grantome.com/grant/NIH/R01-AI145781-01). Other "omics" data that could be used in such an integrated approach are transcriptional data or proteomics.

Future genetic studies will also benefit from precise patient characterization and combination with *M. tuberculosis* genotype (63), in a genome-to-genome approach (64). Also, replication studies in different settings, including relevant covariates, are needed and promising leads need to be trailed in studies focusing on outcome to identify genetic variants important in treatment decisions.

III: Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) is the concept of individualizing drug dosing by measuring the drug concentration in a patient's serum/plasma and adjusting the dose accordingly.

Consequently, TDM is indicated in settings with a risk of low or high tuberculosis drug exposures: presence of altered drug absorption, distribution, metabolism, or excretion (e.g., renal insufficiency), comorbidities that may affect exposure to anti-tuberculosis drugs [HIV infection, diabetes mellitus (65)], drug-drug interactions, and in patients who are slow to respond or with relapsed tuberculosis (66).

Low anti-tuberculosis drug concentrations can lead to inefficient mycobacterial killing, treatment failure, relapse and selection of drug resistance (67), whereas high concentrations may increase the risk of adverse effects (68).

The TDM process comprises (1) obtaining blood samples, (2) measuring drug concentrations, and (3) interpreting the results.

1. The optimal sampling time points and the number of samples depend on the assessed drug. Limited sampling strategies (LSS) predict the most informative sampling time points based on population pharmacokinetic data, i.e., "average" exposures from tuberculosis patients. With the help of LSS, sampling can often be reduced to three or less time points (69, 70).
2. Analysis of the serum/plasma samples is ideally performed with high performance liquid chromatography mass spectrometry (HPLC-MS). HPLC-MS is highly sensitive and specific and allows simultaneous analysis of several tuberculosis drugs in one run of the assay (71).
3. The degree of exposure that is effective depends on the susceptibility of the patient's *M. tuberculosis* strain, as

TABLE 1 | Current standards and future perspectives for Precision Medicine for tuberculosis.

Measure	Current standard	Future perspective for precision medicine	When to apply
Rapid selection of effective anti-tuberculosis drugs to form a treatment regimen.	Rapid molecular Rifampicin-resistance testing followed by phenotypic drug susceptibility testing in liquid and/or solid media cultures.	Rapid, sputum-based automated sequencing of the entire genome of *M. tuberculosis* or of genetic regions (amplicons) of the genome of *M. tuberculosis* where mutations do predict drug resistance. Algorithm-based treatment decisions based on molecular prediction of drug resistance.	At the time of diagnosis.
Supporting the host immunity by endotype-guided decisions for host-directed therapies.	Endotypes of tuberculosis are still not well defined and identification of endotypes to guide host directed therapies is not performed at present.	Optimal testing needs to be discerned, but likely will include a mixture of metabolism, genetic, epigenetic and/or immune functional studies to identify the host endotype. For example, if host immunity was found to be exuberant, then an endotype-specific therapy might consist of a glucocorticoid, NSAID, calcineurin inhibitor (cyclosporin or tacrolimus) or mTOR inhibitor (rapamycin). In contrast, if evaluations identified anergic or exhausted immunity, than immune boosting regimens may be chosen.	Within the first week: of the diagnosis. Should be repeated 4–8 weeks to evaluate dynamic transitions.
Analysis of host genetic variability to predict adverse events and to provide precise therapeutically interventions.	Host genetic markers are currently not identified in clinical practice. At specialized centers and on special request genetic markers such as mutations associated with specific immune deficiencies are evaluated.	Genetic testing before the start of treatment to (1) define dosing of anti-tuberculous treatment and to (2) identify patients susceptible to adverse drug events and (3) to tailor host-directed therapy to the individual patient.	At the time of diagnosis.
Therapeutic drug monitoring.	Only very few centers world-wide perform measurement of drug levels and PK/PD profiles of anti-tuberculosis drugs in routine clinical practice. Even at these centers there are no analytic capacities to monitor several of the 2nd-line anti-tuberculosis drugs.	Regular therapeutic drug monitoring with same-day-results for all anti-tuberculosis drugs for individual dosage adjustments.	For the first month once a week, once a month thereafter throughout the course of treatment.
Individualizing the duration of anti-tuberculosis therapy.	There are no biomarkers available for routine clinical practice to guide clinicians in the decision of the duration of anti-tuberculosis therapy.	Defining the duration of therapy to achieve relapse-free cure based on the measurement of a robust validated biomarker that also identifies patients having the risk for experiencing recurrent disease at early time points during therapy. Ideally, this biomarker should be measurable in a point-of-care test system.	Once a month throughout the course of treatment starting at month 4.

reflected in the minimal concentration that inhibits growth of *M. tuberculosis* (minimal inhibitory concentration—MIC). Dependent on the drug's mode of action, targets are defined as area under the curve (AUC)/MIC, maximal concentration (Cmax)/MIC, or time above MIC (T>MIC). Unfortunately, specific targets are not yet available for anti-tuberculosis drugs and MIC is seldomly determined. Alternatively, drug concentrations are compared to population pharmacokinetic data (66).

Linking NGS data for prediction of minimal inhibitory concentrations (MIC) with TDM data is a very promising concept to provide tailored high-dosage therapies in cases of low level bacillary drug resistance (21, 72). More clinical information will be needed to proof the plausible assumption that mutations in the genome of *M. tuberculosis* corresponding with a mildly elevated MICs to specific medicines can be overcome by a higher-dose administered of these drugs.

There is a disparity between tuberculosis prevalence and available resources for its treatment. TDM is highly resource-intensive with limited reimbursement available to offset cost. As health care providers avoid the costs of purchase and maintenance of equipment without reimbursement, very few centers have implemented TDM in their routine clinical practice. Ideally, results should be available and interpreted within days in order to effectively adjust therapies. New sampling techniques (73, 74), development of automated analytical techniques (immuno-assays) and *in-vitro* models that help to substantiate target values (75) will facilitate its roll-out. TDM integrates information on drug pharmacokinetics and mycobacterial susceptibility to ensure efficacy and prevent toxicity. It could improve the use of currently available drug therapy and individualized high-dose treatments may even overcome some forms of resistance.

IV: Biomarker Based Treatment Decisions

An ideal treatment monitoring test would have a 1–2 day turn-around, be available and implementable in resource constrained settings and could accurately identify when tuberculosis therapy can be terminated to minimize excess treatment. Molecular tests such as the GeneXpert (Cepheid, USA) or the line-probe assays (Hain Life Sciences, Nehren, Germany) are commercially available as rapid diagnostic methods that permit drug-resistance prediction for important first- and second-line drugs (76, 77). As an alternative, transrenal DNA, and lipoarabinomannan

(LAM) detection in urine have been described to diagnose active tuberculosis in people living with HIV (78–80). Screening for tuberculosis with a LAM urine assay in African people living with HIV and low CD4 count lead to a significant decrease in mortality (81). A novel assay with increased sensitivity may even have a larger impact for the management of HIV-associated tuberculosis (82). As an alternative, the detection of mycobacterial DNA from stool samples of patients with tuberculosis highly correlated with sputum-based diagnostic results and were also able to identify patients at risk for experiencing therapy failure (83).

Immunological assays could improve outcomes in populations in whom diagnosis is very difficult, such as children in whom reports of the T-cell activation marker (TAM-TB) test suggest a sensitivity and specificity of ∼83 and 97%, respectively (84). Complex analysis of transcriptomic studies from whole blood have indicated RNA signatures to be associated with future active tuberculosis although the applicability of such tools in a low or middle income country context is uncertain (85–87). However, a recent systematic review and patient-level pooled meta-analysis concluded that blood transcriptional biomarkers reflect only short-term risk of active tuberculosis and surpass WHO benchmarks only if applied to 3–6-month intervals (88).

In addition, certain computer-aided diagnostic (CAD) tools may be able to identify tuberculosis patients with high accuracy (specificity of 98% and of sensitivity 90%) from digital chest-X-ray images (89).

Mycobacterial culture is the most relevant measure of treatment response, but mycobacterial growth is slow and, as treatment progresses, the time to a positive result increases (90). Here, rapid molecular tests detecting only viable bacteria such as the molecular bacterial load assay (MBLA) may be promising for the future treatment monitoring of tuberculosis patients (91). The MBLA correlates with the time to liquid culture positivity. The advantage is that it is rapid and not compromised by contamination of culture. MBLA test results correlates with disease severity, and provide information on the bactericidal effect of different drugs and drug regimens (91–93). As a rapid test measuring the number of viable organisms in a few hours it has potential to identify failing patients. This could suggest infection with a resistant organism or non-adherence and enable additional investigations or alternatively provide reassurance that the patient is responding appropriately. Operational trials to explore this are now underway.

Phenotypic changes on *M. tuberculosis*-specific blood T cells may be able to inform about treatment efficacy as shown in adults and in children (94, 95). Modern imaging techniques such as PET-CT scans may correlate with treatment responses, but alone were not accurate enough to precisely identify patients with recurrent disease in South Africa (96). Interestingly, certain RNA signatures could predict recurrent disease in tuberculosis patients (97, 98). However, these biomarkers have not been prospectively evaluated and markers that could individualize the duration of therapy are missing so far.

DISCUSSION

With technological advances in the field of diagnostics, analytics, and integration of comprehensive data-sets, tailor-made Precision Medicine for patients with tuberculosis is within reach at centers that operate on the frontier of translational research (**Table 1**). As independent measures, genotypic prediction of phenotypic *M. tuberculosis* drug-resistance based on information of entire bacterial genomes, genotypic and phenotypic identification of immune endotypes and human susceptibility to tuberculosis to individualize HDT, and novel biomarkers guiding physicians for individual treatment decisions are already in place at highly specialized centers and usually under research conditions. The clinical application of some of these innovations needs to become the medical standard. However, as a poverty-related disease, the majority of patients affected by tuberculosis live in resource limited settings. Finding funding and performing operational research on the implementation of precision medicine for tuberculosis in these settings will be one of the great challenges for the future.

AUTHOR CONTRIBUTIONS

All authors made a contribution to the acquisition of the information for the work, critically revised the manuscript for important intellectual content, and gave final approval of the current version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest: JH, MR, and CL have filed a patent for a 22-gene model to predict the end of therapy of TB treatment (EP20158652.6).

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Use of TNF Inhibitors in Rheumatoid Arthritis and Implications for the Periodontal Status: For the Benefit of Both?

Fatima Zamri and Teun J. de Vries *

Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit, Amsterdam, Netherlands

The inflammatory diseases rheumatoid arthritis (RA) and periodontitis show similarities in misbalances of cytokine levels, such as tumor necrosis factor-α (TNF-α). RA has been treated for two decades with TNF inhibitors which are effective by blocking TNF's destructive action. Since RA and periodontitis show similarities in high levels of TNF, the periodontal status of RA patients may improve with the use of anti-TNF therapy. To assess this, a systematic review with special emphasis on duration of therapy was performed to evaluate the effect of anti-TNF-α treatment on the periodontal status of RA patients. Overall, studies showed an improvement in periodontal health with anti-TNF therapy. When analyzed over time (6 weeks to 9 months), it became apparent that initial improvements concerned bleeding on probing (BOP) and gingival index (GI) after therapy duration of 6 weeks. Periodontitis parameters that improved after prolonged treatment were: probing pocket depth (PPD) after 3 months and clinical attachment level (CAL) after 6 months. In conclusion, this systematic review reveals that anti-TNF treatment is therefore not only beneficial for rheumatic joints but also for the gums of rheumatoid arthritis patients. We propose that the sequential tissue recovery due to anti-TNF therapy progresses as follows: 1. block of diapedesis by lowering vessel permeability, 2 fewer leukocytes in the inflamed tissue, and 3. reduced proteolytic activity and subsequent repair of collagen fiber functionality and normalization of osteoclast activity. Clinically, this could lead to a decrease in bleeding on probing and ultimately in an improved clinical attachment level.

Keywords: Osteoclast (OC), TNF - α, inflixmab, etenarcept, Rheumatoid arthritis, Periodontitis

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***Correspondence:**
Teun J. de Vries
teun.devries@acta.nl
orcid.org/0000-0001-6464-5172

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INTRODUCTION

Both periodontitis and rheumatoid arthritis are inflammatory diseases. Periodontitis is caused by an inflammatory response to microbes and bacterial toxins, eventually leading to destruction of supporting periodontal tissues around the teeth (1). Rheumatoid arthritis (RA) manifests primarily as a persistent synovial inflammation which causes damage to articular cartilage. If not treated in time, the underlying bone is also affected (2). There are quite a few similarities between RA and PD.

Both diseases show similarities in the prevailing cytokines within the tissues. Overexpression of TNF is at stake in both diseases and causes an imbalance in cytokine levels and therefore damage of soft tissues, progressing to bone, where osteoclasts are further activated by TNF-α (2–4). Furthermore RA patients are prone to develop periodontitis, possibly due to an increase of circulating TNF levels and/or deteriorated motor skills needed for oral hygiene maintenance as a result of damage in the joints. On the other hand, the relationship could also be reverse: periodontitis could cause inflammation of joints induced by periodontal pathogens that enter the circulation due to periodic and frequent oral bleeding. Periodontal pathogens have been detected in inflamed joints of RA patients (5, 6).

Tumor necrosis factor alpha (TNF-α, from here the common term TNF will be used) is a pro-inflammatory cytokine produced in occurrence of inflammation by cells like macrophages and monocytes (7). It is a hallmark cytokine of the pro-inflammatory immune response. TNF can bind to two different receptors, namely TNFR1 and TNFR2 (p75 TNFR). Binding to each receptor sets different signaling cascades in motion that can lead to apoptosis, differentiation, proliferation and migration of cells causing an inflammatory reaction (8). On the other hand TNF plays an inducing role in bone resorption by attributing to the receptor activator of Nf-kB Ligand (RANKL)-signaling pathway by directly activating osteoclast precursor cells (9, 10). TNF sets a natural immune response in motion in reaction to an infection. However in high concentrations it can cause side effects such as a non-tempered inflammatory reaction, increase in osteoclast precursors and osteoclast formation resulting in bone resorption (11–14). Levels of TNF are associated with less favorable indices of the periodontal parameters such as bleeding on probing (BOP), probing pocket depth (PPD), and clinical attachment level (CAL) (15).

TNF inhibitors are used clinically to counterbalance the high TNF levels accounting for joint inflammation, hereby preventing TNF tissue damage in RA. TNF inhibitors have been available for inflammatory diseases like rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, ulcerative colitis and Crohn's disease since 1998. The presently available TNF inhibitors are infliximab, adalimumab, golimumab and etanercept and certolizumab pegol. These inhibitors have an immunoglobin (Ig) structure in common. Infliximab has a 65% similarity to human IgGs, and golimumab and adalimumab have the highest similarity with human IgG. Etanercept is a recombinant fusion drug existing of TNF p75 receptor and the fc component of human IgG1. Anti-TNF reduces inflammatory reaction of the body by blocking TNF-α, hence preventing it to bind on its receptor (TNFR1 or TNFR2). However a prolonged use of TNF inhibitors has a few possible side effects like: hepatotoxicity, malignity, greater risk for infection, immunogenicity and cutaneous reactions.

Heart failure is a contraindication for anti-TNF therapy (16). High levels of TNF- α are correlated with cardiac remodeling resulting eventually in disruption of the cardiac function (17, 18). Therefore high TNF-alfa concentration is a risk for heart failures. Use of TNF inhibitors cause a decrease in TNF- α levels and can so prevent cardiac remodeling and heart failures. Recent studies have shown that TNF inhibitors can have this protective effect (18). However, various studies have shown that cardiac function can be disturbed in patients on TNF inhibitors and are therefore a contraindication in patients with heart failure. To have a better understanding of the effect of the possible protective effect of TNF inhibitors in cardiovascular disease more studies are required.

In vitro and in vivo studies have already proven that the use of anti-TNF (infliximab and etanercept) inhibits osteoclast formation (12, 13, 19, 20). Since periodontitis is manifested as breakdown of alveolar bone, treatment with anti-TNF might have similar positive effects for the periodontium as for the inflamed joints of RA patients. For periodontitis, its effect could be two-fold: lowering of soft-tissue break-down and an inhibition of bone degradation.

Previous work summarizing the effectiveness of anti-TNF therapy in RA and ankylosing spondylitis (AS) has suggested a a positive spin-off for the periodontium (1, 21, 22). No study thus far, however, has assessed how the duration of anti-TNF therapy improves the specific periodontal parameters. The aim of this study is specifically to assess the effectiveness of anti-TNF treatment on possible improvement of specific periodontal parameters. With such a novel approach, we hope to link the blocking of TNF to clinical and therefore biological events at the level of the periodontal tissues.

METHODS

Literature Search

For the literature search a methodological approach was adopted by using PRISMA (Preferred Reporting Items for Systematic Reviews and Meta- Analyses). This method was used to reduce bias in literature selection by the authors.

The electronic search was carried out in the following databases: Embase, Pubmed and Web of Science. Studies up to January 2020 and publications in English were included in this search. The keywords used for the search were: "periodontitis," "periodontal diseases," "periodontal," "adalimumab", "etanercept", "infliximab", "certolizumab pegol", "antibodies tumor necrosis factor- alpha," and "TNF-alpha inhibitors" (**Table 1**), including the search filters 'Humans' and 'English' resulted in excluding suitable publications; therefore, these filters were not used. However, further on we restricted our search to articles written in English only.

Screening and Selection

To select suitable publications a set of inclusion and exclusion criteria was used. All in vitro and animal studies were excluded. Cohort studies, case reports, randomized controlled trials, and longitudinal studies in English were included. Furthermore, only studies that tested at least one of the clinical periodontal parameters (probing depth, BOP, and clinical attachment loss) or radiographic parameters were included. Outcomes of clinical trials compared the effect of anti-catabolic medication on

TABLE 1 | Search query TNF-alpha inhibitors.

Search	Query
#1	Periodontitis OR periodontal diseases OR periodontal
#2	Adalimumab OR etanercept OR infliximab OR certolizumab pegol OR antibodies tumor necrosis factor- alpha OR TNF-alpha inhibitors
#3 (#1 AND #2)	(periodontitis OR periodontal diseases OR periodontal) AND (adalimumab OR etanercept OR infliximab OR certolizumab pegol OR antibodies tumor necrosis factor alpha OR TNF-alpha inhibitors)

positive or negative effects on the periodontium. Articles that did not meet this outcome were excluded.

All titles and abstracts of the publications from the electronic search were screened by two reviewers (FZ and TV) and discussed. When mutual agreement between the two reviewers could not be reached or when the suitability of the publication was questionable the full article was examined by both. The selected literature was divided into the different follow-up periods and presented in tables summarizing the effect of the medications on the following periodontal parameters: plaque index (PI), gingival index (GI), BOP, PPD, and CAL. PI is a score where supragingival plaque is recorded on four or six sites of each tooth. The score defines the absence or presence of plaque by marking it with a positive or negative score. Accumulation of these scores finally results in the PI, which varies from 0 till 100%. The GI can be used to evaluate the condition of the gingiva and is scored at four sites around the teeth. The score varies from 0 to 3, a score of 0 for a normal gingiva and 3 for a severe gingival inflammation (23). BOP is the percentage of bleeding points around the teeth. It is scored at 6 sites around the tooth and can vary from 0 to 100%. PPD is the measurement from the gingival margin to the bottom of the pocket. A measurement of more than 3 mm indicates clinical attachment loss. CAL is defined as the distance from the cemento-enamel junction (CEJ) to the bottom of the pockets.

RESULTS

The electronic research that was carried out resulted in a total of 1,571 articles. After manually removing the duplicates a total of 1,209 publications remained. An additional 1 record was found through manually screening reference lists of articles that were found. All the 1210 articles were manually screened for suitability by reading the titles and abstracts. This resulted in a full text read of 23 articles of which 10 publications were excluded with reasons like: no full text available, no clear description of the used materials and methods, or use of new medication during follow-up period. Finally, 13 publications were included in this review (**Figure 1**). Results and methods of publications discussing the effect of anti-TNF therapy on periodontal parameters (PI, GI, BOP, PPD, and CAL) are presented in **Table 1**. Summarization is in chronological order of publication and was divided in smaller groups depending on the follow-up period. In order to provide a complete impression, tables will also show when examiners in the study were blinded or calibrated.

The Effect of Anti-TNF Treatment in Case-Control Studies

The first group of studies that will be discussed is the group with no follow-up assessment which consisted of 3 studies (**Table 2**). Studies in this group compared patients on anti-TNF therapy to control groups, without baseline assessment before start of anti-TNF therapy. Mayer et al. (24) compared periodontal parameters in RA patients with anti-TNF therapy (mean of 26 months) and in RA patients without therapy. GI, BOP, and CAL were significantly lower in patients using infliximab. When RA patients on anti-TNF therapy (mean of 26 months) were compared to a control group, patients with periodontal disease, without RA or another systematic inflammatory disease and no anti-TNF therapy, BOP and CAL showed also significantly lower parameters. Mayer et al. (25) conducted a similar study with infliximab where RA patients using anti-TNF therapy (RA+) were compared with patients having autoimmune diseases (rheumatoid arthritis, psoriatic arthritis, and systemic sclerosis). The RA+ group showed a statistically significant decrease in periodontal parameters GI, BOP, and PPD. This study also made a comparison between RA patients that used anti-TNF therapy (RA+) and healthy subjects. Also the comparison was made with patients without RA or any other autoimmune diseases. This study showed that there was no statistically significance between the groups in PI, GI, BOP, and PPD (25). Schiefelbein et al. studied the effect of anti-TNF by comparing RA patients that have been using anti-TNF for at least 12 months with non-RA patients. Patients using anti-TNF had a significant lower BOP. CAL showed no statistically significant change (26).

The following results describe studies on TNF inhibitors used for 30 days, 6 weeks, 3 months, 6 months and 9 months. Results are described and compared to baseline measurements and potentially give insight in which parameter improves over time.

30 Days Anti-TNF May Improve PI and GI

Üstün et al. carried out a longitudinal study of 30 days assessing the effect of infliximab and adalimumab in RA patients with periodontitis. 30 Days of anti-TNF-α therapy increased GI significantly while PI, BOP, PPD, and CAL did not change significantly over this time (**Table 2**).

Even in RA patients without periodontitis anti-TNF resulted in an increase of GI (27).

6 Weeks of Anti-TNF May Improve PI, GI, and BOP

Table 2 describes the findings of the study of Ortiz et al. that evaluated the effect of the usage of anti-TNF on periodontal

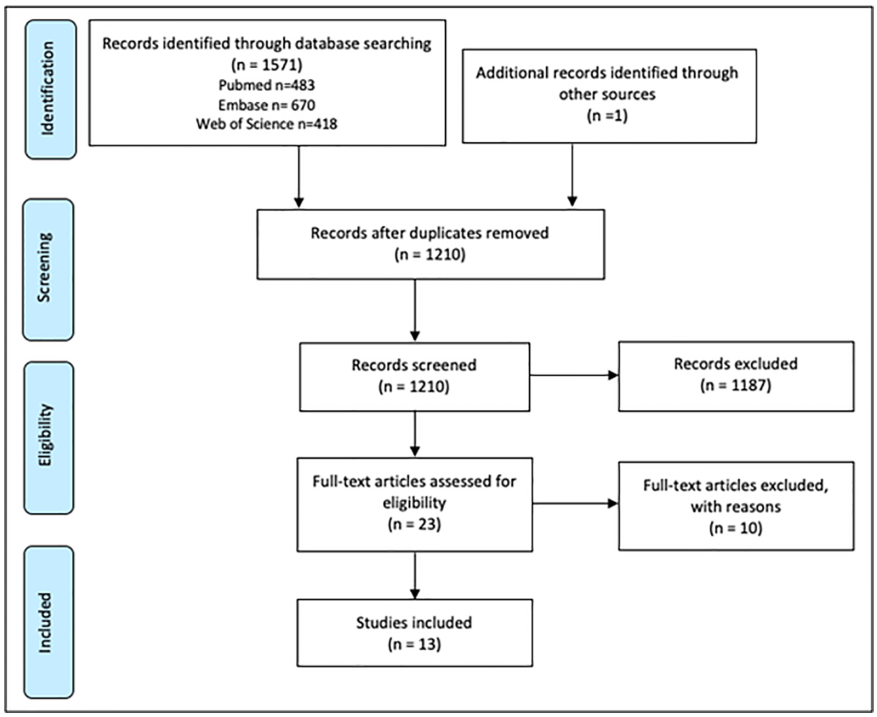

FIGURE 1 | Literature search, strategy, and results.

treatment with a follow-up of 6 weeks. Patients on anti-TNF therapy (infliximab, etanercept or adalimumab) were compared to patients not receiving this therapy resulting in improvement of GI, BOP, and CAL when periodontal treatments were conducted. Anti-TNF therapy without periodontal treatment however did not result in improvement of any of the periodontal parameters (28). Another study investigated the effect of anti-TNF medication by administration of etanercept in RA patients for 6 weeks resulted in a significant reduction of GI and BOP while the oral hygiene index (OHI) remained unchanged (29).

3 Months of Anti-TNF Improves GI, BOP, and PPD

Studies with a follow-up period of 3 months are presented in **Table 2**. One of these studies is by Kobayashi et al. (30) and described the effect of adalimumab on periodontal parameters in a group of RA patients (n = 20). Assessments were performed at baseline and after 3 months of administration of adalimumab. Anti-TNF medication decreased GI, BOP and PPD. However no significant changes were observed for CAL. In 2015 Kobayashi et al. (31) carried out another study where the effect of anti-TNF [infliximab (IFX), etenarcept (ETN), adalimumab (ADA), or golimumab] on periodontal parameters was assessed after 3 and 6 months. After 3 months of anti-TNF administration GI, BOP and PPD improved significantly.

6 Months of Anti-TNF Further Improved All Periodontal Parameters Reported

After 6 months (**Table 2**) the same significant decrease in GI, BOP, and PPD was observed (29).

Though not all parameters were analyzed in all studies it is apparent that significant improvements have been reported for all parameters. Savioli et al. tested the effect of the use of TNF inhibitors (IFX, ETN, and ADA) in RA patients with and without periodontitis. In both groups there was no significant improvement in BOP, PPD or CAL. However in the RA group with periodontitis the PI decreased significantly ($p < 0.03$) (32). Fabri et al. researched the effect of IFX, ETN, and ADA on the periodontal parameters in ankylosing spondylitis (AS) (n = 15) and RA patients (n = 15). AS patients showed an improvement in PPD ($p = 0.01$) and CAL ($p = 0.04$) while the other parameters (PI and gingival bleeding index) remained stable ($p > 0.05$). In the RA group no significant changes were observed (33). Two other studies in this group reached similar results. Ancuta et al. examined RA patients (n = 96) and Iordach et al. examined AS patients (n = 86). Both studies showed a statistical significant decrease after 6 months of anti-TNF therapy in GI, BOP, PPD, and CAL (34, 35). Since the literature has reported most frequently on the 6 months after treatment, the quantitative effects of before and after treatment on the 5 periodontal parameters is summarized in **Figure 2**. Seven studies evaluated the effect on periodontal parameters after administration of anti-TNF treatment for 6 months. The quantitative analysis is shown in **Figure 2**. Three of the seven studies (31, 33, 34) showed a significant decrease in BOP, four studies showed a significant decrease in PPD [(33), in AS patients] and in RA patients (31, 34), and these three studies showed a significant decrease in CAL despite the stability of the PI. This indicates that changes in BOP,

TABLE 2 | Studies assessing the effect of TNF inhibitors on periodontal parameters.

Authors	TNF inhibitor	Subjects	Assessment	PI	GI	BOP	PPD	CAL
Varied, no baseline								
Mayer et al. (24)	IFX	RA+ (n = 10) compared to RA without anti-TNF-α (n = 10)	1 moment (1 calibrated examiner)	NS p > 0.05	↓ (sig.) p = 0.0042	↓ (sig.) p = 0.0146	NS p = 0.0554	↓ (sig.) p = 0.0273
		RA+ (n = 10) compared to healthy patients (n = 10)	1 moment(1 calibrated examiner)	NS p > 0.05	NSp > 0.05	↓ (sig.) p = 0.0146	NS p = 0.0554	↓ (sig.) p = 0.0273
Mayer et al. (25)	IFX	RA+ (n = 12) compared to AI (RA, PA and SSc) (n = 36)	1 moment(2 calibrated examiners)	NS p = 0.0548	↓ (sig.) p = 0.0005	↓ (sig.) p = 0.0002	↓ (sig.) p = 0.0001	–
		RA+ (n = 12) compared to healthy patients (with PD, no AI's or anti-TNF-α) (n = 12)	1 moment(2 calibrated examiners)	NS p > 0.05	NSp > 0.05	NSp > 0.05	NS p > 0.05	–
Schiefelbein et al. (26)	ETN (n = 2)ADA (n = 5) Golimumab (n = 6)	RA with PD on anti-TNF-α for >12 months (n = 13) versus non RA with PD (n = 13)	1 moment(1 calibrated examiner)	NSp = 0.182	–	↓ (sig.)p = 0.045	↓ (NS)p = 0.068	NSp = 0.134
30 days follow up								
Üstün et al. (27)	IFX (n = 9)ADA (n = 7)	RA patients with PD (n = 10)	BL + after 30 days(1 examiner)	NS p = 0.779	↑(sig.) p = 0.016	NS p = 0.067	NS p = 0.413	NSp = 0.326
6 weeks follow up								
Ortiz et al. (28)	IFXETNADA	RA patients on PDT and anti-TNF-α (n = 10)	BL + after 6 weeks(1 calibrated examiner)	↓ (sig.)p < 0.01	↓ (sig.)p < 0.01	↓ (sig.)p < 0.01	↓ (sig.)p < 0.01	↓ (sig.) p < 0.05
		RA patients on anti-TNF-α (n = 10)	BL + after 6 weeks(1 calibrated examiner)	NSp > 0.05	NSp > 0.05	NSp > 0.05	NSp > 0.05	NSp > 0.05
		Anti TNF (n = 10) versus no anti-TNF-α (n = 10)	BL + after 6 weeks(1 calibrated examiner)	NS p = 0.37	↓ (sig.) p < 0.001	↓ (sig.) p < 0.001	NS p = 0.107	↓ (sig.) p < 0.001
Kadkhoda et al. (29)	ETN (n = 36)	RA patients (n = 36)	BL + after 6 weeks	NS *p = 0.860	↓(sig.)p = 0.036	↓(sig.) p = 0.049	NS p = 0.126	–
3 months follow up								
Kobayashi et al. (30)	ADA (n = 20)	Patients with RA (n = 20)	BL + after 3 months(2 calibrated examiners)	NS p = 0.12	↓(sig.) p = 0.002	↓(sig.) p = 0.003	↓(sig.) p = 0.002	NSp = 0.42
Kobayashi et al. (31)	IFX (n = 6)ETN (n = 9) ADA(n = 19) Golimumab (n = 6)	RA patients treatment with anti-TNF-α (n = 40)	BL + after 3 months(1 examiner calibrated + masked)	NS	↓(sig.) p < 0.017	↓(sig.) p < 0.017	↓(sig.) p < 0.017	NS
6 months follow up								
Kobayashi et al. (31)	IFX (n = 6)ETN (n = 9) ADA(n = 19) Golimumab(n = 6)	RA patients treatment with anti-TNF-α (n = 40)	BL + after 6 months(1 examiner calibrated + blinded)	NS	↓(sig.) p < 0.017	↓(sig.) p < 0.017	↓(sig.) p < 0.017	NS
Savioli et al. (32)	IFX (n = 15)ETN (n = 2) ADA (n = 1)	RA patients with PD (n = 8)	BL + after 6 months(1 blinded examiner)	↓(sig.) p = 0.03	–	NS p = 0.50	NS p = 0.25	NSp = 0.84
		RA patients without PD (n = 10)	BL + after 6 months(1 blinded examiner)	NSp = 0.27	–	NSp = 0.95	NS p = 0.36	NSp = 0.91
Fabri et al. (33)	IFXETNADA	AS patients and PD (n = 7)	BL + after 6 months(1 blinded examiner)	NS p = 0.21	–	NS §p = 0.25	↓(sig.) p = 0.01	↓(sig.) p = 0.04
		Patients with RA and PD (n = 7)	BL and + 6 months(1 blinded examiner)	NS p = 0.076	–	NS §p = 0.118	NS p = 0.381	NSp = 0.36
Ancuta et al. (34)	IFX, ETN and ADA	RA patients (n = 96)	BL and after 6 months	NS p > 0.05	NSp > 0.05	↓(sig.) p < 0.05	↓(sig.) p < 0.05	↓(sig.) p < 0.05
Iordache et al. (35)	IFX, ETN, ADA andGolimumab	AS patients (n = 86)	BL and after 6 months	NS p > 0.05	NSp > 0.05	↓(sig.) p < 0.05	↓(sig.) p < 0.05	↓(sig.) p < 0.05
9 months follow up								
Pers et al. (36)	IFX (n = 20)	RA patients with PD (n = 9)	BL + after 9 months(1 blinded examiner)	NS p > 0.05	↑(sig.) † p < 0.05	↑(sig.) ‡p < 0.05	NS p > 0.05	↓(sig.)p < 0.05

*RA, rheumatoid arthritis patients; RA+, rheumatoid arthritis with anti-TNF-α therapy; AI, Autoimmune disease; PA, psoriatic arthritis; SSc, systemic sclerosis; AS, ankylosing spondylitis; PD, periodontitis; PDT, periodontal treatment; GI, gingival index; PI, plaque index; BOP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment level; IFX, infliximab, ETN, etanercept; ADA, adalimumab; BL, baseline, n, number; sig., statistically significant; NS, not statistically significant, *Oral hygiene index (OHI), §Gingival bleeding index (GBI), †Modified gingival index (MGI), ‡Papillary bleeding index (PBI).*

Green: significant improvement, Red: significant worsening.

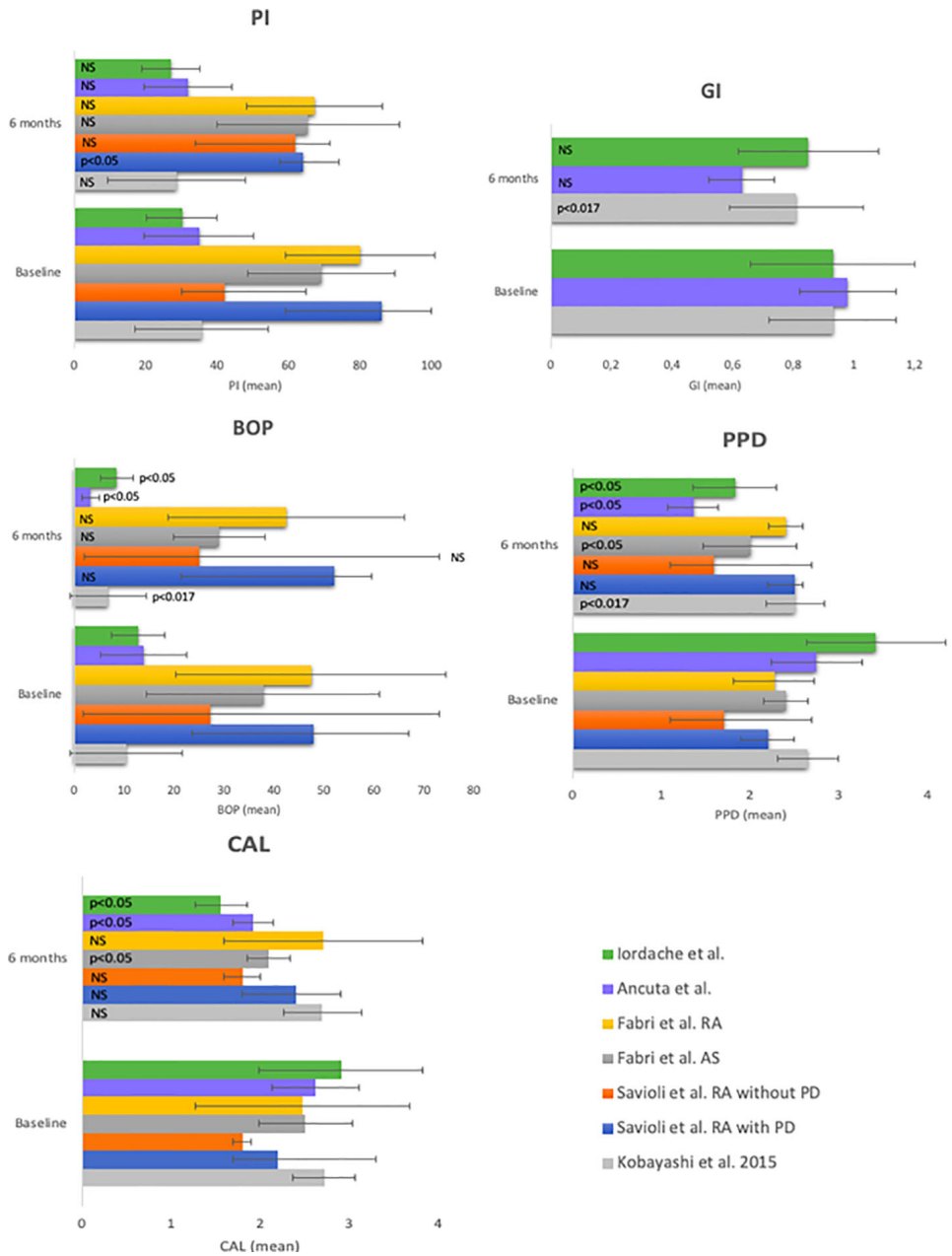

FIGURE 2 | Periodontal parameters at baseline and at 6 months of anti-TNF-α treatment. PI, plaque index; GI, gingival index; BOP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment level.

PPD, and CAL are not a result of a change in oral hygiene, but rather from systemically tempering TNF.

9 Months of Anti-TNF and Worsening of GI BOP and Improvement of CAL

The final study with a follow up period of 9 months is Pers et al. (**Table 2**). This longitudinal study included 9 patients with RA and periodontal disease. These patients were assessed before anti-TNF therapy with IFX and after 9 months. Results showed that the modified GI (MGI) and papillary bleeding index (PBI) increased, therefore worsened, significantly after 9 months of IFX therapy, while CAL decreased significant. No significant changes were observed for PI and PPD (36).

Efficacy of TNF Treatment for RA

In order to control for the efficacy of anti-TNF for the original application, to minimize the rheumatoid arthritis induced

inflammation burden, various parameters can be measured. Nearly all studies, except Pers et al. measured the Disease Activity Score 28 (DAS28) (37) and cytokine levels in serum or gingival crevicular fluid (GCF). DAS 28 measures the activity of disease by evaluating the number of swollen and tender joints out of 28. Other parameters were the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) and a self-assessment of health using the visual-analog scale (VAS) (37).

By evaluating changes in these parameters, the effectiveness of TNF-inhibitors in reducing inflammation can be evaluated. These findings are shown in **Table 3**.

Most of the studies showed a decrease in DAS28, serum CRP levels or TNF levels and thus the anti-inflammatory effect of anti-TNF therapy. A few studies did not show a decrease in these inflammatory parameters. Schiefelbein et al. showed no decrease in serum CRP levels when RA patients on anti-TNF were compared to RA patients without anti-TNF (26). Ortiz et al. also showed no improvement in DAS28 and serum TNF levels in RA patients after 6 weeks on anti-TNFtherapy (28). Furthermore, Savioli et al. showed no significant change in DAS28 and serum CRP levels after 6 months of anti-TNF in RA patients with periodontitis (32).

DISCUSSION

This literature review presents an overview of the possibly beneficiary effect of TNF inhibitors used in rheumatoid arthritis on periodontal parameters, with special emphasis on which parameters improve over time. The effectiveness of TNF inhibitors in periodontal therapy can be assessed when measuring the anti-inflammatory effects of TNF inhibitors on periodontal parameters of the examined subjects. Measurement of DAS28 is essential in all studies in order to establish effect on joints due to TNF inhibitors in patients. Studies have shown that it takes 2 weeks of anti-TNF (infliximab, etanercept, adalimumab, or golimumab) administration to observe a clinical response in RA and AS patients (38–41). Therefore, a study design with a period shorter than 2 weeks is likely not to

TABLE 3 | Studies assessing the effect of TNF inhibitors on rheumatologic parameters and cytokine levels.

Authors	Subjects	Assesment	DAS28	Serum CRP levels	Serum TNF-α levels	GCF TNF-α levels
Mayer et al. (24)	RA+ (n = 10) compared to RA without anti-TNF-α (n = 10)	1 moment(1 calibrated examiner)	NS p > 0.05	–	–	↓(sig.) p = 0.04
Mayer et al. (25)	RA+ compared to AI (RA, PA and SSc)	1 moment(2 calibrated examiners)	–	–	–	↓(sig.) p = 0.002
Schiefelbein et al. (26)	RA with PD on anti-TNF-α for >12 months (n = 13) versus non RA with PD (n = 13)	1 moment(1 calibrated examiner)	–	NS p = 0.310	–	–
Üstün et al. (27)	RA patients with PD (n = 10)	BL + after 30 days(1 examiner)	↓(sig.) p = 0.001	↓(sig.) p = 0.02	–	–
Ortiz et al. (28)	RA patients on PDT and anti-TNF-α (n = 10)	BL + after 6 weeks(1 calibrated examiner)	↓(sig.) p < 0.005	–	↓(sig.) p < 0.001	–
	RA patients on anti-TNF-α (n = 10)	BL + after 6 weeks(1 calibrated examiner)	NS p > 0.005	–	NS p = 0.2	–
Kadkhoda et al. (29)	RA patients (n = 36)	BL + after 6 weeks	–	–	–	↓(sig.) p = 0.04
Kobayashi et al. (30)	Patients with RA (n = 20)	BL + after 3 months(2 calibrated examiners)	↓(sig.) p < 0.001	↓(sig.) p < 0.001	↓(sig.) p < 0.001	–
Kobayashi et al. (31)	RA patients treatment with anti-TNF-α (n = 40)	BL + after 3 and 6 months(1 examiner calibrated + masked)	↓(sig.) p < 0.017	↓(sig.) p < 0.017	↓(sig.) p < 0.017	–
Savioli et al. (32)	RA patients with PD (n = 8)	BL + after 6 months(1 blinded examiner)	NS p = 0.11	NS p = 0.55	–	–
	RA patients without PD (n = 10)	BL + after 6 months(1 blinded examiner)	↓(sig.) p = 0.04	↓(sig.) p = 0.01	–	–
Fabri et al. (33)	AS patients and PD (n = 7)	BL and + 6 months(1 blinded examiner)	–	↓(sig.) p = 0.03	–	–
	Patients with RA and PD (n = 7)	BL and + 6 months(1 blinded examiner)	↓(sig.) p = 0.01	↓(sig.) p = 0.008	–	–
Ancuta et al. (34)	RA patients (n = 96) RA patients (n = 96)	BL and after 6 months	↓(sig.) p < 0.05	↓(sig.) p < 0.05	↓(sig.) p < 0.05	–
Iordache et al. (35)	AS patients (n = 86)	BL and after 6 months	↓(sig.) *p < 0.05	↓(sig.) p < 0.05	–	–
Pers et al. (36)	RA patients with PD (n = 9)	BL + after 9 months(1 blinded examiner)	–	–	–	–

*RA, rheumatoid arthritis patients; RA+, rheumatoid arthritis with anti-TNF-α therapy; AS, ankylosing spondylitis; AI, Autoimmune disease; PA, psoriatic arthritis, SSc, systemic sclerosis; PDT, periodontal treatment; PD, periodontitis; DAS28, disease activity score; CRP, C-creative protein; sig., statistically significant; NS, not statistically significant *ASDAS28 ankylosing spondylitis disease activity score.*

Green: significant improvement, Red: significant worsening.

show any improvement in periodontal or any inflammatory parameter. In line with this, the study with the shortest standardized period in this review was Üstün et al. with therapy for 30 days which did not show a response of the periodontal parameters (27). This indicates that a possible periodontal response requires more time than an initial dampening of inflammation. Probably, an adequate response for periodontal tissues is shown after 3–4 months of anti-TNF therapy in most patients (42, 43). Ortiz et al. studying the effect of anti-TNF without periodontal treatment for 6 weeks is one of the studies where probably a too short time window was used to observe any adequate response. However, particularly in this study, also DAS28 or serum TNF levels (28) were not altered. This suggests that there is no overall response in these patients to anti-TNF therapy, because either the administration period was too short or there was no response to the medication (primary inefficiency) which is seen in 30–40% of RA patients when using TNF inhibitors (44). On the other hand Ortiz et al. show that patients with RA on anti-TNF therapy demonstrate a significant improvement in GI, BOP, and CAL when anti-TNF therapy was combined with periodontal therapy. Ortiz et al. proved that there is a significant additional value when anti-TNF combined to PDT is used to treat periodontitis compared to patients only receiving periodontal treatment or only using anti-TNF-α. In another study where anti-TNF was administered for 6 weeks, it was found that periodontal parameters (GI and BOP) and cytokine levels (GCF TNF levels) were significantly decreased (29). These changes indicate that anti-TNF leads to less inflammation and some improvement of periodontal parameters, but does not result in a decreased PPD. These observed changes might be a result of anti TNF therapy.

The time point 6 months is the most widely studied, therefore, we summarized only this time point quantitatively (**Figure 2**). Although these 6 studies with 7 different patient groups show significant and beneficiary responses, one should bear in mind that high standard deviations persist which occasionally overlap severely. Besides this, some improvements maybe significant, but many of them are rather moderate.

When summarizing the findings of this study, the periodontal status of rheumatoid arthritis patients receiving anti-TNF administration benefitted from this treatment, especially when analyzed from 6 weeks up to 6 months after start of intervention. However, an administration period of more than 9 months resulted in aggravation of the gingival condition and increase of GI. In rheumatoid arthritis, studies have shown that over time 30–40% of the patients may lose response to anti-TNF treatment (secondary loss of response) (44). Secondary loss of response can be developed by treatment with biological agents. These agents, like anti-TNF therapy can induce an immune response causing formation of ADAs (anti-drug antibodies) resulting in a neutralizing effect of this drug (45). The results by Pers et al., may hint that this also accounts for losing benefit for the periodontal status in these patients. No cytokine levels were measured in this study so nothing can be said about the responsiveness to therapy of the patients in the study group. Scheifelbein et al.[3] proved that long-term administration (>12 months) still had a positive effect on cytokine levels, and periodontal parameters. Mayer at al. in 2009 (24) and in 2013 (25) also proved that in RA patients with a mean anti-TNF administration of 26 months both GCF cytokine levels and periodontal parameters improved significantly.

As earlier described responsiveness to the anti-TNF therapy has been evaluated per study group in the included studies and not per individual. Lack of responsiveness to anti-TNF therapy might be correlated to ineffectiveness of improving periodontal parameters. Therefore, it is of importance to evaluate if lack of responsiveness in one subject translates in the same subject to a lack of improvement in periodontal parameters. When identifying predictive parameters, anti-TNF therapy could be applied in therapies combating inflammatory diseases. Alternatively, in cases of non-responsiveness, IL-6-R inhibitor treatment could be an option, as described by Kobayashi and coworkers (31). Shortcomings of all studies discussed here, are the relatively small number of included subjects and scarce comparisons between the effects of the different TNF inhibitors. Therefore, the results in this review might show an underestimation or overestimation of some TNF-inhibitors. More research is required to evaluate the effect of each different TNF inhibitor to asses which one is the most effective for the periodontal paramenters. Moreover, no studies have been carried out with healthy individuals with periodontitis on anti-TNF therapy. Such a study could be considered, although one should take into ethical clinical consideration whether one should expose relatively healthy people to a drug that makes the immune system less alert.

Pers et al. (36) showed a significant reduction in CAL but not in PPD. To explain this observation clinical attachment gain, coronal migration of periodontal support, must have occurred and/or the alveolar bone must have increased in height. However this last explanation does not support the findings of Cenk Durmuslar et al. (46), where rats showed no bone regeneration after use of infliximab, which is also in line with Ferreira-Junior et al. (47), where studies in rats proved that infliximab did not affect bone remodeling. Since only one study in this review suggests that there might be a beneficial effect in bone remodeling by the use of anti-TNF there is not enough evidence to support this claim.

Based on the findings described in this paper and based on general cell biological knowledge of TNF-α (8), we propose the following model, showing the sequence of events that may take place in the periodontium when anti-TNF therapy is effective. TNF has been correlated to processes such as increased PMN rolling on endothelial cells, concomitant with increased classical adhesion molecules for this process such as ICAM-1 and P-selectin, a process that was counterbalanced by anti-TNFtreatment (48). More recently, the effect of TNF on diapedesis showed that especially PMNs migrate into tissues when TNF is high (49). Since an effect on BOP is one of the first aspects that improves, we would like to suggest that vessel permeability is first of all restored, together with a downregulation of endothelial adhesion molecules for diapedesis, such a ICAM-1 (**Figure 3**, numbers 1 and 4). This then results in a decrease in leukocytes that migrate into the tissue (**Figure 3**, numbers 2 and 5). Since these leukocytes are potential producers of TNF-α, that

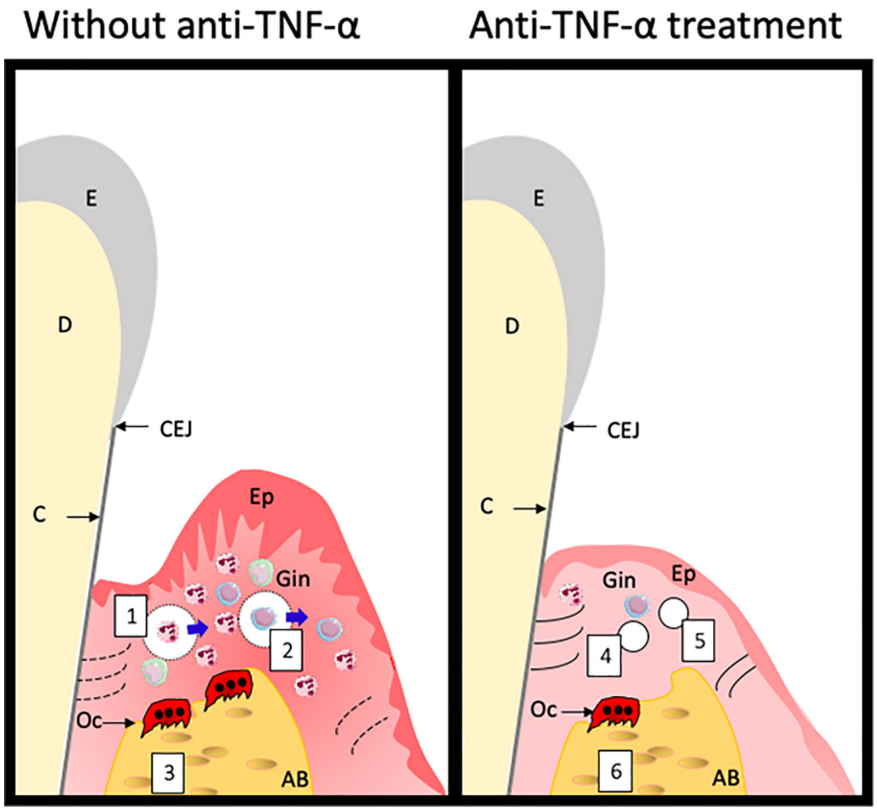

FIGURE 3 | Hypothetical changes of the periodontium following anti-TNF treatment. Treatment with anti-TNF-α leads to overcoming the TNF-α associated cellular processes such as vasodilatation and permeability for leukocytes (1), proteolysis by this excess of leukocytes of extracellular matrix components, depicted as dotted lines (2) and activation of osteoclasts (3). Anti-TNF-α treatment may lead to less dilated and less permeable blood vessels (4), less proteolytic activity, restoring the extracellular matrix production (5) and tempering osteoclast formation and activity. Clinically, this all leads to less bleeding on probing, and an improvement in attachment which is observed by a decreased PPD and CAL. E, Enamel; CEJ, cemento-enamel-junction; C, cementum; D, dentin; Ep, epithelium; Gin, Gingiva; Oc, osteoclast; AB, alveolar bone.

may induce proteolytic activity (50) and signaling towards osteoclasts (9, 10, 12, 18), the overall switch is towards anabolic activities (**Figure 3**, numbers 3 and 6). Clinically, this leads to improved PPD and CAL (**Figure 3**). Proof of these hypothetical changes should come from histological assessments, but these are scarce in the field of periodontitis.

This overview presented promising results that anti-TNF therapy is beneficial in the treatment of both RA and periodontitis by improving periodontal parameters of RA patients. One would like to be able to predict which patients with both RA and periodontitis would benefit from which TNF inhibitor. Future research is needed to elucidate this further.

AUTHOR CONTRIBUTIONS

Idea for the systematic review: TV. Literature search and first draft: FZ. Writing: FZ and TV. Completion: FZ and TV. All authors contributed to the article and approved the submitted version.

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The freely available Servier Medical Art catalog (https://smart.servier.com) was used for immune cells in **Figure 3**.

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Insulin Modulates Inflammatory Cytokine Release in Acute Stages and Augments Expression of Adhesion Molecules and Leukocytes in Lungs on Chronic Stages of Paracoccidioidomycosis

Felipe Beccaria Casagrande[1], *Sabrina de Souza Ferreira*[1], *Emanuella Sarmento Alho de Sousa*[1], *João Pedro Tôrres Guimarães*[1], *Lavínia Maria Dal'Mas Romera*[2], *Fernando Henrique Galvão Tessaro*[1], *Sandro Rogério de Almeida*[2], *Stephen Fernandes de Paula Rodrigues*[3] *and Joilson O. Martins*[1]*

[1] *Laboratory of Immunoendocrinology, Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences of University of São Paulo (FCF/USP), São Paulo, Brazil,* [2] *Laboratory of Mycology, Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences of University of São Paulo (FCF/USP), São Paulo, Brazil,* [3] *Laboratory of Vascular Nanopharmacology, Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo (ICB/USP), São Paulo, Brazil*

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Stefan Niemann, Research Center Borstel (LG), Germany

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***Correspondence:**
Joilson O. Martins
martinsj@usp.br

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Type 1 *diabetes mellitus* (T1D) is caused by partial destruction of the insulin-producing beta cells in the pancreas and is a major issue for public health care worldwide. Reduced or impaired immunological responses, which render patients more susceptible to infections, have been observed in T1D, and this dysfunction is often related to a lack of insulin in the blood. Paracoccidioidomycosis is an important systemic mycosis endemic in Latin America. To evaluate the effects of T1D on this fungal infection and the modulatory effects of insulin, we induced diabetes in C57Bl/6 male mice (alloxan, 60 mg/kg), infected the mice (Pb18, 1 x 10^6 cells), and treated the mice with neutral protamine Hagedorn (NPH) insulin (2 IU/600 mg/dL blood glucose). Twenty-four hours after infection, infected diabetic mice showed reduced secretion of interferon (IFN)-γ and interleukine (IL)-12 p70 compared to infected nondiabetic controls. On the 45th day of infection, infected diabetic mice presented higher IFN-γ levels, a higher tumor necrosis factor (TNF)-α:IL-10 ratio, and lower adhesion molecule expression levels than nondiabetic mice. In the *in vitro* experiments, alveolar macrophages from diabetic animals showed reduced phagocytic activity compared to those from control animals at 4, 12, and 24 h. In infected diabetic mice, treatment with insulin restored IL-12 p70 levels at 24 h of infection, reduced IFN-γ levels and the TNF-α:IL-10 ratio at 45 days, and restored vascular cell adhesion molecule (VCAM)-1 expression in pulmonary blood vessels, and this treatment reduced the diminished phosphorylation of extracellular signal-regulated kinases (ERK) and increased nuclear factor-kappa-B(iκb)-α and jun amino-terminal kinases (JNK) p46

levels in infected nondiabetic mice. In addition, insulin promoted increased phagocytic activity in the alveolar macrophages of diabetic mice. These data suggest that T1D mice are more susceptible to Pb18 infection and that insulin modulates this inflammation in diabetic mice by augmenting the expression of adhesion molecules and leukocytes in the lungs and by reducing chronic inflammation.

Keywords: macrophages, type 1 diabetes, inflammation, Paracoccidioidomycosis, systemic mycosis, fungal infection, vascular cell adhesion molecule-1 expression

INTRODUCTION

Diabetes mellitus comprises a group of metabolic disorders characterized by a relative lack and/or reduced response to endogenous insulin on target cells (1), leading to metabolic and vascular complications related to hyperglycemia that affect several organs and systems (1, 2). It is estimated to affect more than 425 million patients worldwide (1, 2). Type 1 *diabetes mellitus* (T1D) is described as the partial or complete destruction of insulin-producing beta cells from Langerhans islets in the pancreas, and this causes hyperglycemia.

Among complications related to T1D, the impairment of immunological responses to diverse inflammatory stimuli has been widely observed in diabetic patients, and this includes reduced production of the inflammatory cytokine interleukin (IL)-1β in *Mycobacterium tuberculosis* infection (3) and increased susceptibility to skin, bone, and joint infections and to fungal diseases (4). Some of the impairments found in people with diabetes are reproduced in animal models of T1D, such as reduced expression of intercellular adhesion molecule (ICAM)-1, reduced migration of leukocytes to inflammatory sites, diminished secretion of tumor necrosis factor (TNF)-α (5), reduced phagocytic activity of diabetic rat neutrophils (6), and reduced bactericidal activity of Paneth cells in diabetic mice (7). Experimental treatment with insulin was observed to promote different outcomes regarding immunological responses under different inflammatory conditions (4–7). However, there are still few studies relating insulin to the chronic pattern of inflammation observed in some fungal infections.

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by *Paracoccidioides* sp. fungi; PCM was first observed in 1908 and described in 1930 (8), and it ranks as the top cause of hospitalizations among systemic mycoses (9). PCM occurs when a host inhales the mycelial form of the fungus originating from the soil, and several variables determine the severity of the disease, such as the inhaled fungal load, strain virulence, and host immunocompetence (9). As occurs in many systemic mycoses, phagocytosis and destruction of infectious agents by cells of the immune system is highly important for the efficient and asymptomatic resolution of PCM infection (10). An efficiently developed or preexisting T helper 1 (Th1) response and the secretion of cytokines that activate phagocytic activity on macrophages and stimulate T cells, interferon (IFN)-γ, IL-12, and TNF-α are often observed to contain the infection during the subclinical period (11–13). On the other hand, a deficient or inhibited Th1 response is observed in individuals in which PCM has progressed to the chronic stage, suggesting higher susceptibility to the agent (11, 14).

T1D is an important disease that has a significant socioeconomic impact worldwide; it is ranked among the main public health issues (1, 2). At a smaller scope, hospitalizations and mortalities resulting from decompensated PCM have a high occurrence in Latin America, especially in Brazil (15). Although the association between T1D and PCM in literature is not strong, both are considered highly undiagnosed/underreported diseases (1, 8), and our studies show an important rise in susceptibility for a more severe case of PCM in T1D mice, possibly resulting from an overall reduced protection against the pathological agent (16), and that insulin partially restored cellular inflammation indexes. In this work, we aimed to study the effects of insulin on leukocyte presence and activity in a mouse model of T1D infected with *P. brasiliensis*.

METHODS

Animals

This study used pathogen-free mice of the C57Bl/6 strain, and it was performed in accordance with the guidelines accepted by the Brazilian National Council for Control of Animal Experimentation (CONCEA) of the School of Pharmaceutical Sciences (FCF) in the University of São Paulo (USP) (the project is registered under the permit CEUA/FCF/512). Surgical procedures were performed under anesthesia (ketamine hydrochloride 90 mg/kg and xylazine hydrochloride 10 mg/kg, respectively; Sespo, Brazil), and all care was taken in order to minimize animal suffering. This work used 49 mice, distributed into 3–6 animals/group, all male and weighing 18–22 g in the beginning of the experimental period, in which they were maintained in a controlled environment at 22°C and a 12-h light-dark cycle. Chow and water were available ad libitum throughout the experiment.

Diabetes Mellitus

An alloxan-induced model was used to induce T1D in mice (16, 17). Briefly, mice received intravenous injections of alloxan monohydrate [60 mg/kg of animal dissolved in 100 μL of sterile saline solution (NaCl 0.9%); Sigma-Aldrich, United Kingdom]. Mice from control groups were injected with 100 μL of sterile saline instead. The parameters confirming diabetic state were obtained 10 days after the injection by measuring blood glucose (Accu-Chek Advantage II, Roche Diagnóstica, São Paulo, Brazil) in samples collected from mouse tails. Animals presenting blood glucose higher than 300 mg/dL were considered diabetic.

Paracoccidioides brasiliensis

An isolate of a *Paracoccidioides brasiliensis* strain known to be virulent (16, 18) (Pb18; Laboratory of Mycology, School of Pharmaceutical Sciences, University of São Paulo, Brazil) was used for this study. The cultures were grown in Sabouraud's semisolid medium at 37°C with weekly subculture. Colonies of Pb18 yeasts were collected in sterile phosphate buffer solution (PBS; 18 mM Na_2HPO_4, 3 mM $NaH_2PO_4H_2O$, and 140 mMNaCl in Mili-Q water) and vortexed for 1 min before filtration with a 40 μm cell strainer (BD Biosciences) thrice. The concentration of yeasts was counted in a Neubauer hemocytometer and adjusted to a standard before inoculation.

Pb18 Infection

On the 10th day, after confirmation of T1D, mice were anesthetized (ketamine/xylazine hydrochloride) and, upon unconsciousness being confirmed, were inoculated with 1 x 10^6 yeast cells in 50 μL sterile PBS *via* intratracheal injection. Noninfected groups received 50 μL sterile PBS instead by the same procedure.

Insulin Treatment

Eight hours after infection by Pb18, mice from the insulin-treated groups received neutral protamine Hagedorn insulin (NPH; Eli Lilly, São Paulo) by subcutaneous injections for evaluation of its effects on a 24-h infection. Alternatively, 33 days after infection, mice received one injection a day at 6 pm during the 12 days preceding the experiment, completing a 45-day infection. In both cases, dose was calculated according to blood glucose levels presented by mice (2 IU insulin/600 mg/dL of blood glucose) (16, 17). A graphical schema for the protocol can be observed in **Figure 1**.

Organs Harvesting and Cell Count

After euthanasia 24 h or 45 days following infection, lungs were harvested and washed in sterile PBS and then disrupted manually in PBS using a Potter-Elvehjem tissue grinder. The large particulate material was removed by filtration with a 70-μm cell strainer, and the filtrate was centrifuged. Supernatant was stored in -80°C for posterior analysis while cells in the pellet were suspended in PBS supplemented with 3% fetal bovine serum (FBS). The leukocyte concentration of each sample was assessed using Neubauer hemocytometer slides and Turk's solution, and results were presented in cells/mL.

Quantification of cytokines

IFN-γ, IL-10, TNF-α, and IL-12 p70 were quantified in lung macerate supernatant with the use of a Cytometric Bead Array Mouse Inflammation Kit (BD, Biosciences). Assays were performed according to the manufacturer's instructions, and samples were measured by flow cytometer (FACSCanto II, BD Biosciences), where results were analyzed using BD CBA Software (BD, Biosciences). Results are expressed in pg/mL.

RNA Extraction and Real-Time PCR

Total RNA from lung homogenates was extracted following the protocol described in Cold Spring Harbor Protocols (19). cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (lot 00376305, ThermoFisher Scientific) and real-time PCR was executed with the following primers: *il12, tnfα, il6, il10, il4*, and *tgfβ* (all from Exxtend®; More information about the primers can be found in the **Supplementary Material**) at Applied Biosystems *StepOnePlus*™ Real-Time PCR System (ThermoFisher Scientific). Relative gene expression was calculated by comparative threshold cycle (Ct) and expressed relative to the controls (ΔΔCt method).

Quantification of Signaling Molecules *via* Western Blot

Lungs of mice were harvested after 45 days of Pb18 infection and disrupted with RIPA lysis buffer. The homogenates had their protein determined (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific Inc., IL) and calculated into 50-μg samples. Proteins were separated *via* electrophoresis in polyacrylamide gel and then transferred to nitrocellulose membranes (Amersham Biosciences Corp., NJ, USA). Following 1 h blocking (5% nonfat dried milk in Tris-buffered saline Tween (20 mM Tris, 150 mM NaCl, 1% Tween 20; TBST), membranes were washed with TBST thrice and stayed overnight at 4°C in primary antibodies against the target molecules (p38 MAPK, P-p38 MAPK, ERK 1/2 MAPK, P-ERK 1/2 MAPK, JNK, iκB-α, TLR-2, pAKT, PKC-α). All antibodies were purchased from Cell Signaling Biotechnology (MA, USA). After 3 washing steps, membranes were incubated for 1 h with antirabbit secondary antibody (1:10,000; Abcam). β-actin (1:50,000, 1 h; Sigma) was

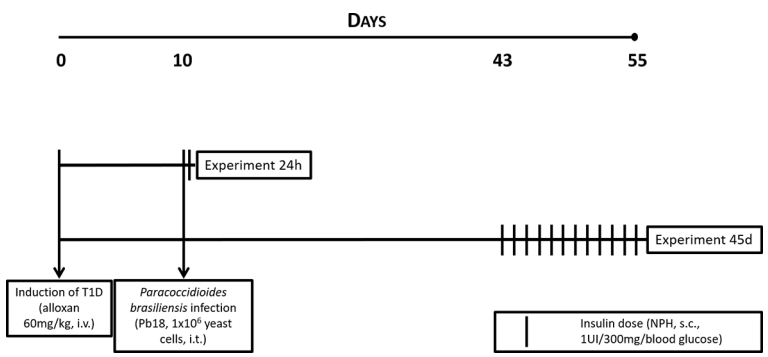

FIGURE 1 | Experimental protocol for T1D, infection with *P. brasiliensis*, and treatment with NPH insulin in mice.

used as loading control. More information about the antibodies can be found in the **Supplementary Material**. For development, we used chemiluminescence detection in an Amersham Imager 680 blot and gel imager (Amersham, Buckinghamshire, UK), and densitometric analysis of the bands was performed using Image Studio software (LI-COR Biosciences, Lincoln, Nebraska, USA). Results are represented by densities of each band divided by density of its respective loading control.

Quantification of Adhesion Molecule

For this study, we quantified expression of VCAM-1 by the immunohistochemistry assays. Briefly, the superior right lobes of the lungs were stored in 10% formaldehyde and dehydrated by baths in crescent concentrations of ethanol (70% to 100%) before being imbedded in paraffin. Transversal sections were collected in silanized slides and fixated with formaldehyde. In the moment of analysis, slides are bathed on xylene twice for 5 min, the sections are hydrated in baths in decreasing concentrations of ethanol (twice in 100%, then 95% and 80%), 3 min each. Antigen retrieval, blocking of peroxidases, and blocking for unspecific binding were performed using reagents and instructions provided by the kit EnVision FLEX+ (Dako, Denmark), followed by overnight incubation with primary antibody (1:50; Santa Cruz Biotechnology) at 4°C in a humid chamber. After washings, sections were incubated for 1 h with a secondary antibody (conjugated to horseradish peroxidase) provided by the kit and, following a washing step, incubated with substract containing 3,3-diaminobenzidine for 8 min. Slides were washed and mounted with organic resin (Entellan, MERK) and then observed in a light microscope Leica DMLFS (Leica Microsystems, Wetzlar, Germany), using a DFC300FX camera (Leica Microsystems) to capture the images.

Five pictures showing at least one blood vessel were taken from each sample, and quantification of the mean values of staining per area was performed with ImageJ software.

Infection Index and Phagocytosis CFU

Alveolar macrophages (AM) were obtained by bronchoalveolar ex vivo lavage from diabetic (alloxan 60 mg/kg in NaOH 0.9%; 10 days) and nondiabetic (NaOH 0.9%; 10 days) mice. The fluid was centrifuged at 259 G/10 min and the cells in the pellet suspended in RPMI-1640 (Gibco) medium. Volumes containing 5 x 10^5 AM were transferred to each well containing glass cover slides, maintained in an incubator at 37°C and 5% CO_2 for 1 h for adhesion, and then washed with warm PBS and incubated with RPMI-1640 supplemented with 2% FBS (Vitrocell, São Paulo, Brazil) for 18 h.

To evaluate the phagocytic activity, nonadhered cells were washed out with warm PBS, and the adhered cells were incubated (RPMI-1640. 10% FBS, penicillin 100 μg/mL, gentamicin 100 μg/mL, and streptomycin 100 μg/mL) in the presence or absence of insulin [insulin from bovine pancreas, reconstituted and prepared according to manufacturer's instructions (0.005%) Sigma-Aldrich; Catalog number i6634]. Volumes containing 5 x 10^5 yeasts of Pb18 were added to the wells, followed by incubation for 4, 12, or 24 h in a CO_2 incubator to allow adhesion and ingestion of the fungus. After incubation, cover slides were washed and stained with hematoxylin and eosin and then mounted on microscopy slides with organic resin and observed in a light microscope. Each well had 300 macrophages analyzed, and the infection index was calculated following the formula (II = Internalized Yeast/Total Macrophages*100) (20).

Assays of CFU were performed to quantify the yeast internalized by the macrophages. AM cultures (RPMI-1640, 10% FBS, penicillin 100 μg/mL, gentamicin 100 μg/mL, and streptomycin 100 μg/mL) with or without insulin (insulin from bovine pancreas, 0.005% Sigma) were infected with Pb18 yeasts (5 x 10^5 yeasts) in sterile PBS and incubated (37°C, 5% CO_2). After 4, 12, or 24 h of interaction, medium was removed, and wells were washed three times to remove nonadhered yeasts. Macrophages were ruptured using 200 μL 0.1% Triton (Sigma-Aldrich, USA) in cold PBS. The cell lysate was plated on brain hearth infusion (BHI; KASVI) semisolid medium and incubated for 20 days at 37°C, after which the recovered colonies were counted.

Data Analysis

The data were evaluated by analysis of variance (ANOVA) followed by the Tukey-Kramer posttest for multiple comparison using GraphPad Prism 7.0 software. Data are here represented by mean values ± standard error mean (SEM), and values $p<0.05$ were considered significant for this study.

RESULTS

Characterization of T1D Model

In our previous publication, we showed that the injection of alloxan caused a significant reduction in weight gain (mean ± SEM; control, 2.1 ± 0.4 g in 10 days and 6.8 ± 0.8 g in 55 days; diabetic, 0.5 ± 0.3 g in 10 days and 3.1 ± 1.5 g in 55 days; $p<0.001$) and sharply elevated blood glucose levels (control, 195 ± 9 mg/dL in 10 days and 167 ± 5% nbsp;mg/dL in 55 days; diabetic, 489 ± 18 mg/dL in 10 days and 506 ± 66 mg/dL in 55 days; $p<0.001$) (16).

Evaluation of Inflammation in the Lungs at Early Stages of PCM (24 h)

In our previous publication, we showed that the injection of alloxan caused a significant reduction in weight gain (mean ± SEM; control, 2.1 ± 0.4 g in 10 days and 6.8 ± 0.8 g in 55 days; diabetic, 0.5 ± 0.3 g in 10 days and 3.1 ± 1.5 g in 55 days; $p<0.001$) and sharply elevated blood glucose levels (control, 195 ± 9 mg/dL in 10 days and 167 ± 5 mg/dL in 55 days; diabetic, 489 ± 18 mg/dL in 10 days and 506 ± 66 mg/dL in 55 days; $p<0.001$)

In the analysis of samples obtained 24 h after infection, we observed no significant difference between diabetic and nondiabetic mice in the leukocyte count in the lungs (**Figure 2A**). Compared to infected control mice, infected diabetic mice showed reduced levels of the proinflammatory cytokines IFN-γ and IL-12 p70 (**Figures 2B, C**; $p=0.0223$ and $p=0.0337$, respectively).

Treatment with a single dose of insulin resulted in a significant 3-fold increase in leukocytes in the lungs as well as a more than 10-fold increase in secreted IL-12 p70 levels in diabetic animals (**Figures 2A, C**; $p=0.0026$ and $p=0.0426$, respectively). Interestingly, insulin also promoted a reduction in the levels of IFN-γ in infected nondiabetic mice (**Figure 2B**).

AM were obtained from nondiabetic and diabetic mice and then infected *in vitro* with Pb18 yeast to evaluate their infection capacity in the presence or absence of insulin. The growth of colony forming units (CFUs) on cell lysates of the same samples was analyzed to confirm the internalization of the agent. **Figure 3** shows that AM from diabetic animals presented a reduced infection index compared to cells from nondiabetic mice. This difference was observed at 4 h (p=0.0306; **Figure 3A**), 12 h (p=0.0071; **Figure 3C**), and 24 h (p=0.0236; **Figure 3E**). Based on our CFU analysis, the cell lysate of macrophages from nondiabetic mice presented an increased number of colonies compared to that of AM from diabetic mice after 4, 12, and 24 h of interaction (**Figures 3B, D, F**).

The addition of insulin to the culture medium did not change AM from nondiabetic mice but restored phagocytic capacity in AM from diabetic mice in experiments at 12 h (1.7-fold; p=0.0222) and 24 h (1.5-fold; p=0.0452). This increase in yeast internalized by AM from diabetic animals in the presence of insulin was significant at 12 h interaction (**Figure 3D**).

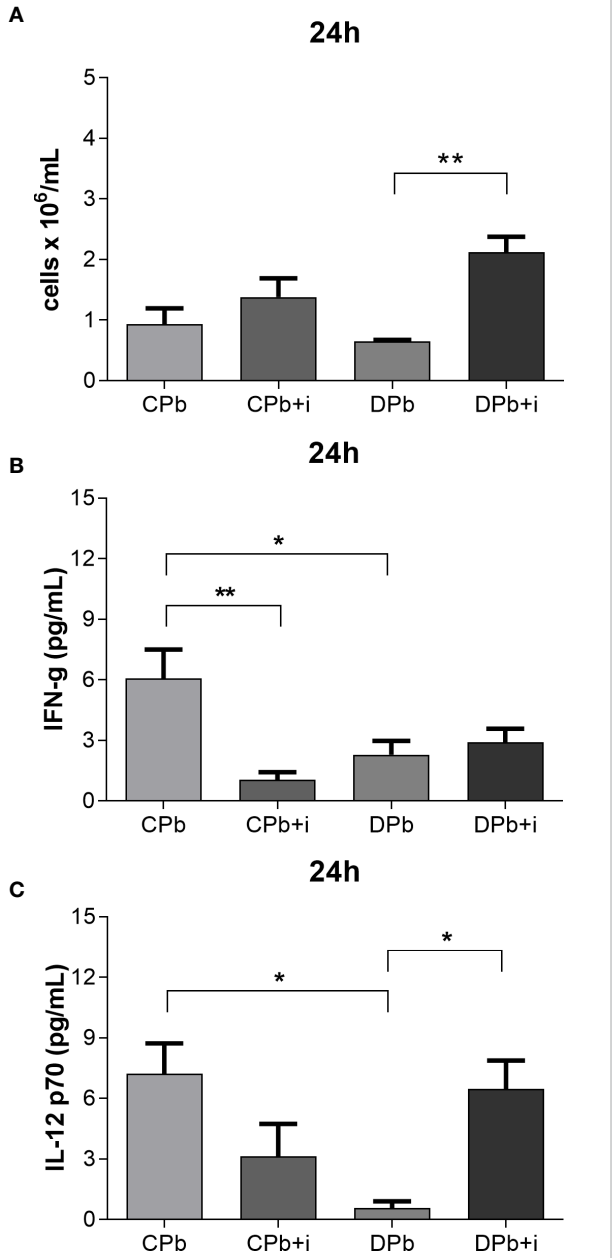

FIGURE 2 | Assessment of inflammation in lungs – 24 h. T1D mice and control mice infected with Pb18 yeasts with and without one dose of insulin 8 h after infection. Samples were obtained 24 h after infection. **(A)** Leukocyte count in lungs; **(B)** IFN-γ in lungs; **(C)** IL-12 p70 in lungs. Bars represent mean value ± SEM. *$p < 0.05$; **$p < 0.01$.

Evaluation of Inflammation in the Lungs at Late Stages of PCM (45 Days)

Obtained at later stages of the infection, 45-day samples showed no difference in the number of leucocytes present in the lungs of diabetic and nondiabetic mice (**Figure 4A**). Diabetic mice showed a more proinflammatory environment than nondiabetic mice as evidenced by higher IFN-γ levels and a higher TNF-α:IL-10 ratio (**Figures 4B, D**; p=0.0240 and p=0.0356, respectively), whereas the levels of IL-12 p70 did not differ between the groups (**Figure 4C**). Twelve injections of insulin given once a day before the experiment did not affect nondiabetic mice in regard to these parameters although, in diabetic animals, this treatment increased leukocytes (**Figure 4A**; p=0.0183). In addition, insulin treatment reduced IFN-γ levels (2.6-fold; p=0.0463) and the TNF-α:IL-10 ratio (4.4-fold; p=0.0276) in diabetic mice (**Figures 4B, D**).

Blood vessels in lungs harvested after 45 days of infection were stained to quantify the presence of vascular cell adhesion molecule (VCAM)-1 *via* immunohistochemistry. **Figure 5** shows a significant increase in the expression of VCAM-1 in the vessels of nondiabetic infected mice (p=0.0205) but not in diabetic mice. Treatment with insulin did not change VCAM-1 expression in infected nondiabetic animals, but increased the expression of this adhesion molecule in diabetic mice (**Figure 5**; p=0.0211).

Figure 6 shows the results of RT-PCR analysis performed on lungs after 45 days of infection. Compared to infected nondiabetic mice, diabetic mice infected with Pb18 presented higher *il6* and *il4* expression (**Figures 6A, B**; p<0.0001 and p=0.0026, respectively). Treatment with insulin resulted in reduced *il6* expression in infected diabetic mice (**Figure 6A**; p<0.0001) and increased *il4* expression in infected nondiabetic mice (**Figure 6B**; p=0.0389). There were no significant differences in the expression of *tnfα*, *il10*, *il12*, and *tgfβ* between diabetic and nondiabetic mice with or without insulin treatment.

We evaluated the levels of signaling molecules in inflammatory pathways in the lungs *via* Western blot analysis. In samples obtained 45 days after infection, the concentrations of the studied molecules in infected and noninfected mice were not different for both the diabetic and nondiabetic groups. Treatment with insulin, however, resulted in diminished phosphorylation of ERK in infected nondiabetic mice as seen by a decrease in the p-ERK: total ERK ratio (**Figure 7B**; p=0.0460). Insulin treatment also resulted in augmented levels of whole fractions of iκb-α (**Figure 7D**; p=0.0496) and JNK p46 (**Figure 7F**; p=0.0320) in infected nondiabetic mice but did not induce changes in infected diabetic mice. This experimental model did not result in significant

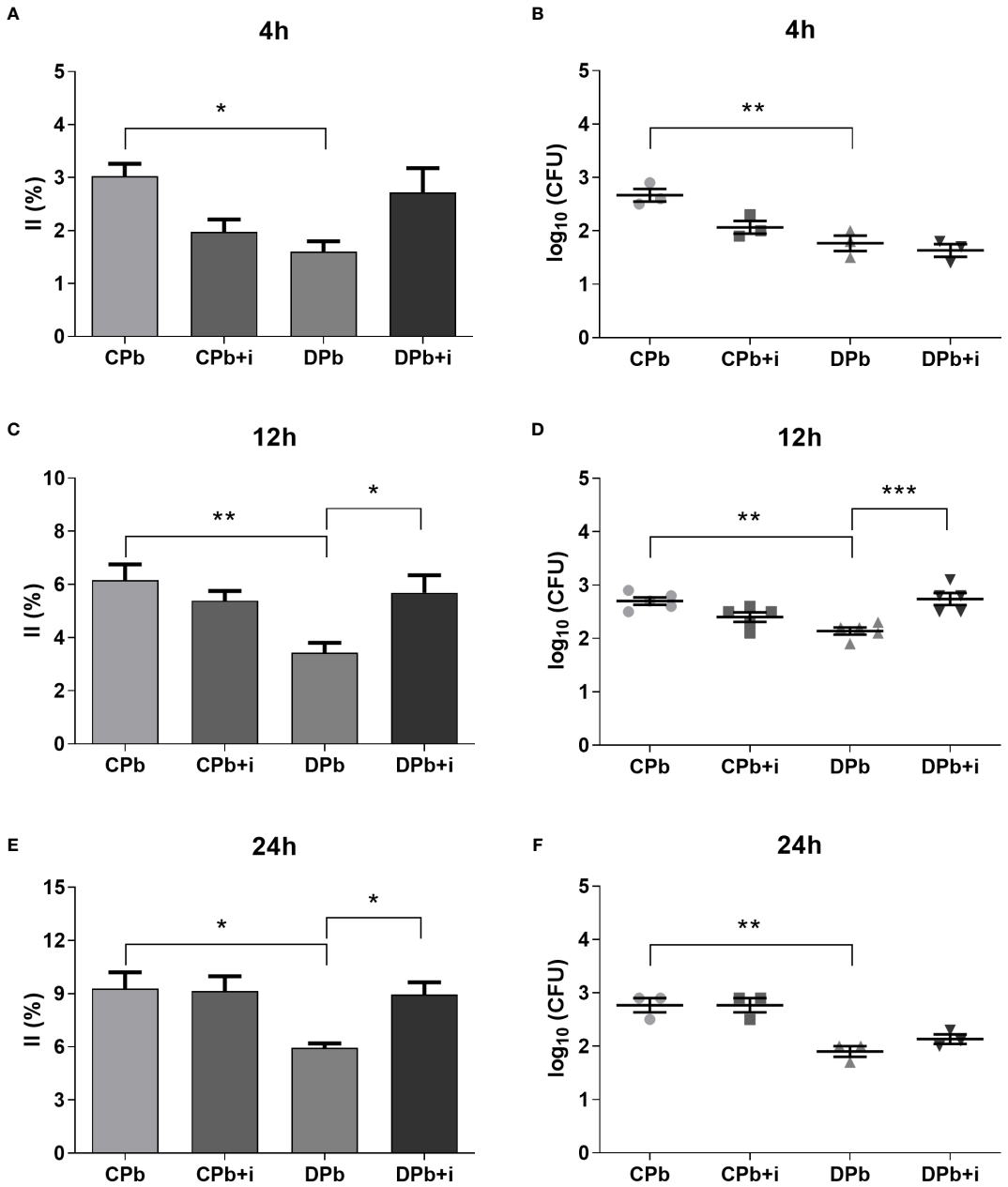

FIGURE 3 | Phagocytic activity *in vitro*. AM from T1D mice and control mice were infected with Pb18 yeasts *in vitro* with or without insulin in the medium. **(A, B)** 4 h after infection; **(C, D)** 12 h after infection; **(E, F)** 24 h after infection. Bars represent mean value ± SEM. II, infection index; CFU, colony forming units. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

alterations in the ratios of p-p38:total p38 and p-p44:total p44 (**Figures 7A, C**, respectively) or in the levels of JNK p54, TLR-2, p-Akt, and PKC-α (**Figures 7E–I**, respectively).

DISCUSSION

The data shown in this study are summarized in **Table 1** and suggest the involvement of insulin in the inflammatory process in mice infected with *P. brasiliensis*, which is a dimorphic fungus endemic in Latin America. Henceforth, we used a model of the relative absence of insulin, alloxan-induced T1D, combined with exogenous insulin treatment and *in vitro* insulin treatment. Previous studies from our group have established a protocol of insulin treatment of alloxan-induced diabetic animals in which the effective dose of insulin able to significantly reverse inflammatory parameters observed in diabetic animals was chosen. Even though this dose only partially reduces the blood glucose, elevated levels of insulin can be observed during the whole time of the experiment (16, 21). Although this dose was able to restore inflammatory parameters in diabetic mice,

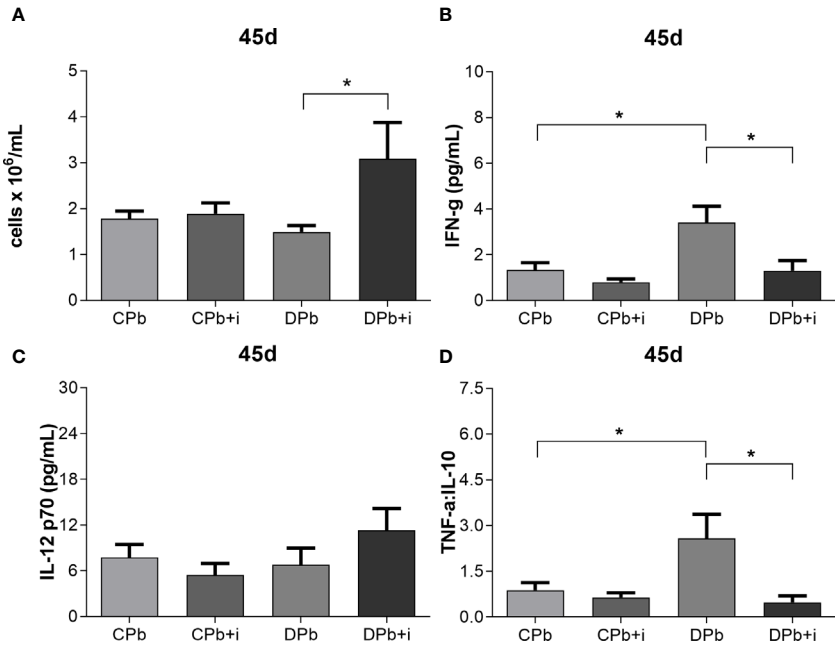

FIGURE 4 | Assessment of inflammation in the lungs at 45 d. T1D mice and control mice infected with Pb18 yeasts with or without daily doses of insulin 12 days before the experiment. Samples were obtained 45 days after infection. **(A)** Leukocyte count in lungs; **(B)** IFN-γ in lungs; **(C)** IL-12 p70 in lungs. **(D)** TNF-α:IL-10 ratio. Bars represent mean value ± SEM. *$p < 0.05$.

it was not sufficient to return glucose levels in diabetics to normal values, and it also had no effect on mortality. Thus, we can associate the effects in insulin-treated mice primarily to the presence of insulin rather than to reduction of glycemia.

Deleterious effects of T1D on the immunological system have been observed before, and studies have associated it with the relative absence of insulin rather than hyperglycemia onset (22, 23). Anjos-Valotta et al. (5) associated T1D with reduced expression of TNF-α and ICAM-1 in TNF-stimulated diabetic rats, and treatment with insulin restored these parameters (5). On the other hand, treatment with insulin has also been related to a decrease in inflammation in diabetic patient as well as the suppression of NF-κB expression and molecules whose gene transcription is dependent on NF-κB, such as monocyte chemotactic protein-1 and -9 and plasminogen activator inhibitor-1 (23, 24). Although most studies in the field consistently agree that T1D causes a higher susceptibility to different types of infections, the modulatory role of insulin seems to differ according to the inflammatory stimulus to which the subject is exposed (3, 5, 25).

For this study, we chose experimental *P. brasiliensis* infection as an inflammatory stimulus due to its epidemiological and socioeconomic relevance to further elucidate the effects of T1D and insulin treatment. Animal models of PCM have been used to better understand the mechanisms and symptoms of PCM, and we chose a mouse strain previously described to have intermediate susceptibility to Pb18 (26).

An effective inflammatory response to Pb18 relies on both innate and adaptive immunity: phagocytosis and destruction of the etiological agent; production and secretion of Th1 proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-12; followed by activation of macrophages, TCD4+ cells, and TCD8+ cells. When the inflammatory response is efficient, the infection is often contained in the subclinical stage (12, 13, 27). In this work, in samples obtained 24 h after infection, we observed less acute inflammation in diabetic animals than in nondiabetic animals with lower levels of IFN-γ and IL-12 p70 in the lungs. In 2001, Benard et al. observed that more severe PCM in an animal model was related to reduced levels of IFN-γ and IL-12, and Kashino et al. (27) associated low levels of IFN-γ with the faster development of PCM in susceptible strains of mice (27). Here, infected diabetic mice treated with insulin presented higher number of leukocytes in the lungs and higher secretion of IL-12 p70 in the lungs, suggesting a more effective response to Pb18 infection.

Phagocytosis and the destruction of pathological agents are crucial to the host's response to several different infections, especially during acute inflammation; these mechanisms represent the main protective mechanisms against most systemic mycosis (28), including PCM, and effective responses leading to phagocytosis and destruction of the yeast cells have been related to faster subclinical clearance and better prognosis (10, 12). The role of macrophages and how they are activated in response to a pathogen are key elements of an appropriate immune response, and the metabolic environment has an important impact on this response. Previous studies showed that lineage (29) and peritoneal macrophages (30) cultured under high glucose conditions tend to present M2-like phenotype characteristics, which would polarize them toward an adaptive rather than an innate immune response, and other

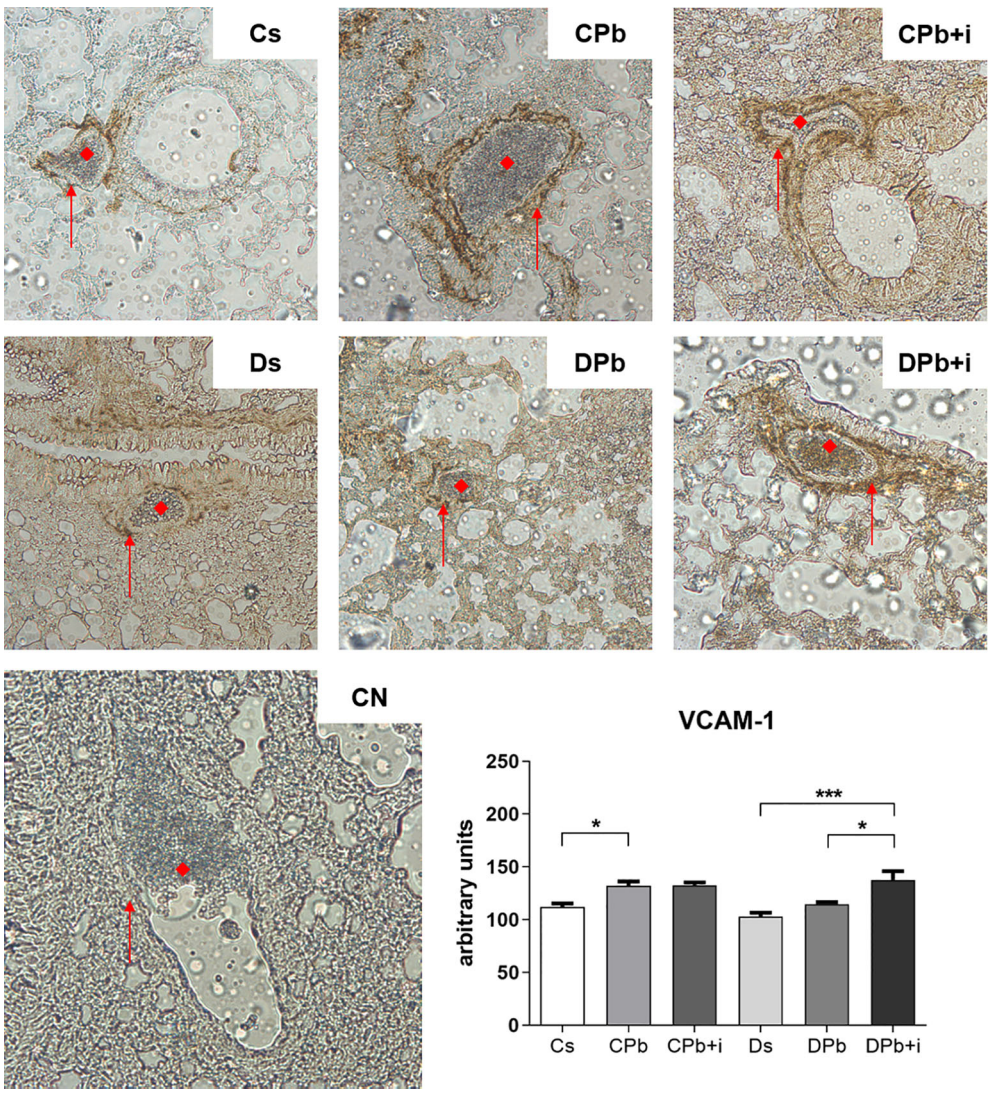

FIGURE 5 | Expression of VCAM-1 in the pulmonary vascular wall. T1D mice and control mice infected with Pb18 yeasts with or without daily doses of insulin 12 days before the experiment. Samples were obtained 45 days after infection. Immunostaining is represented by brown coloration on the vascular wall and is shown by arrows. (◆) Pulmonary blood vessel (magnification, x20). Bars represent mean value ± SEM. *$p < 0.05$; ***$p < 0.001$.

studies relate the impaired phagocytic ability in AM of diabetic rats to reduced phosphorylation of ERK, Akt, and PKC-δ resulting from deficient bonding of leukotrienes to FcγR signaling pathways (31). In addition, Ayala et al. show that high glucose levels appear to modify macrophage behavior, affecting different aspects of diabetic (impaired phagocytic ability, reduced production of reactive hydrogen species, and reduced expressions of TLR-4 on the cell surface) and healthy bone marrow-derived macrophages under the same LPS stimulus, hypothesizing that hyperglycemia leaves a glucose legacy, altering the basal steady state of macrophages (32). More recently, Tessaro et al. (33) also showed that *in vitro* treatment with insulin is able to amplify inflammatory cytokine secretion by bone marrow-derived macrophages from diabetic mice stimulated with LPS by enhancing phosphorylation of MAPK (p42 MAPK, p44 MAPK, p46 SAPK, p54 SAPK) resulting from TLR-4 activation with LPS, and mice deficient of mechanisms related to detection and phagocytosis, such as expressions of TLR-2 and TLR-4, were observed to be more susceptible to PCM (10). In the results of this work, macrophages obtained from T1D mice showed reduced phagocytic activity against Pb18 yeast cells compared to AM obtained from controls, and the presence of insulin in the medium restored phagocytic activity even though insulin treatment *in vivo* in diabetic animals did not alter the levels of IFN-γ. These results could help to explain the high susceptibility presented by T1D mice to Pb18 observed in previous studies (16). These data also corroborate previous findings in animal models as macrophages

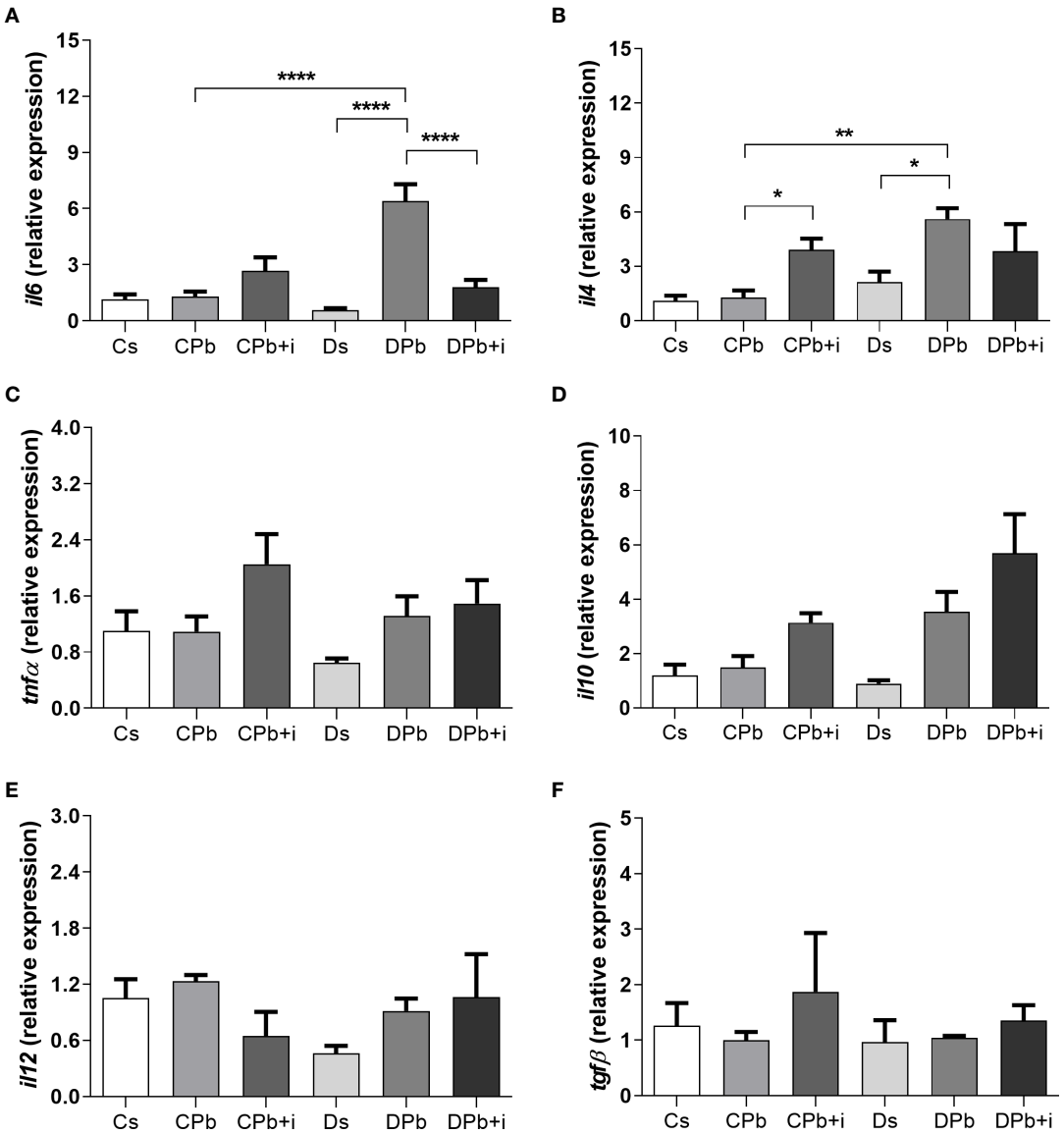

FIGURE 6 | Gene expression in lungs of T1D mice and control mice after 45 days of infection with Pb18 yeasts with and without daily doses of insulin 12 days before the experiment. Expression of **(A)** *il6*; **(B)** *il4*; **(C)** *tnfα*; **(D)** *il10*; **(E)** *il12*; and **(F)** *tgfβ*. Bars represent the mean value ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

from hyperglycemic mice presented reduced phagocytic activity against *Salmonella typhimurium* (34), and diabetic rat neutrophils were also less efficient against *Candida albicans* yeasts (35). These phagocytic dysfunctions in T1D were associated with changes in cell metabolism and in insulin levels. Alba-Loureiro and collaborators' study in 2006 observed that insulin treatment restored phagocytic function in diabetic rat neutrophils (6), whereas Yano et al. (36) observed that treatment with insulin augmented the phagocytic activity of T1D mouse neutrophils against *Staphylococcus aureus* and increased bactericidal capacity. These results provide supporting evidence that a metabolic change caused by insulin could directly affect susceptibility to infections related to T1D.

Moreover, insulin seems to influence in multifaceted ways macrophages from distinct origins. In alloxan-induced diabetes in rats without infection, hyperglycemia and absence of insulin did not change, for example, autophagosome LC3 levels in bronchoalveolar lavage fluid (37). When bone marrow cells were differentiated into M1-like macrophages, those cells derived from diabetic animals without infection have lowered their autophagosome LC3 content. Conversely, cells differentiated into M2-like macrophages have their autophagic LC3 content enhanced. When one of the proteins (Atg12) responsible for conjugating LC3 into the phagosome was screened, it was diminished in the splenic macrophages from red pulp of the diabetic animals without infection

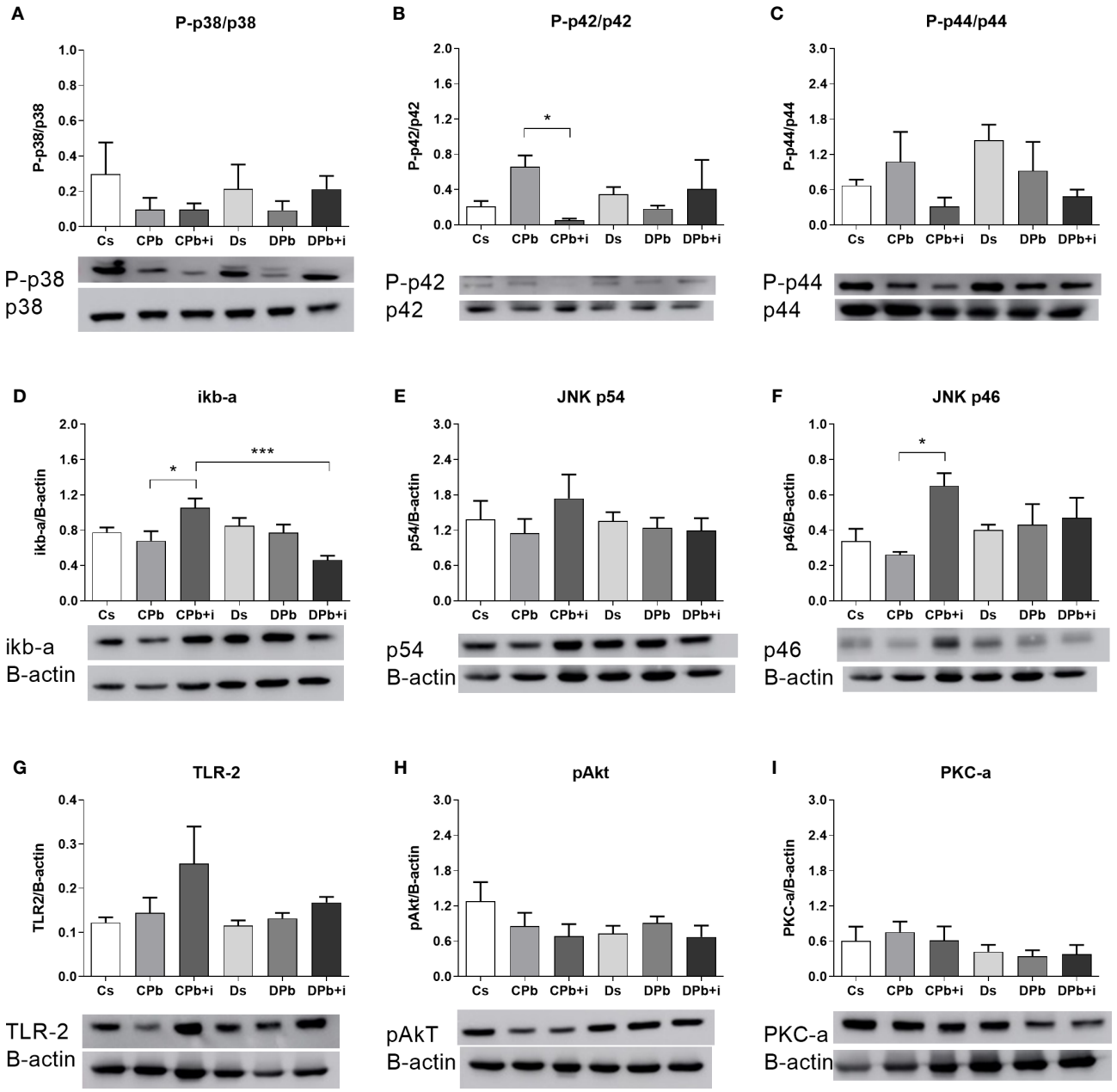

FIGURE 7 | Quantification of signaling molecules in the lungs of T1D mice and control mice after 45 days of infection with Pb18 yeasts, with or without daily doses of insulin 12 days before the experiment. Levels of **(A)** P-p38; **(B)** P-p42 ERK; **(C)** P-p44 ERK; **(D)** iκB-α; **(E)** p54 JNK; **(F)** p46 JNK; **(G)** TLR-2; **(H)** pAkt; and **(I)** PKC-α. Bars represent the mean value ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$.

compared to healthy control. When diabetic rats were treated with insulin, splenic macrophages failed to restore Atg 12 content (37). In addition, the modulatory effects of insulin in chronic inflammations in experimental models of T1D has also been reported. In previous studies using similar models of later stages of PCM in T1D mice, we observed diabetic mice to be more susceptible to PCM than nondiabetic mice, probably due to a reduction in populations of TCD4+ cells, TCD8+ cells, NK cells, and B lymphocytes, which are reportedly important to contain the spread and proliferation of the etiological agent (38, 39). Even though the absolute number of leukocytes in bronchoalveolar lavage fluids did not vary within the groups, the reduction of these populations resulted in augmented fungal loads in the lungs of diabetic mice compared to the lungs of controls (16). In the present work, samples obtained after 45 days of infection revealed that diabetic

TABLE 1 | Summarized data results observed in nondiabetic and diabetic mice infected with Pb18, with and without treatment with insulin.

Effects of Pb18 infection in diabetic mice with and without insulin treatment		
	T1D	**T1D + Insulin**
24 hours – lungs		
Leukocytes number	NS	Higher
IFN-γ levels	Lower	NS
IL-12 p70 levels	Lower	Higher
Infection Index – alveolar macrophages		
4 h	Reduced	NS
12 h	Reduced	Augmented
24 h	Reduced	Augmented
45 days – lungs		
Leukocytes number	NS	Higher
IFN-γ levels	Higher	Lower
IL-12 p70 levels	NS	NS
TNF-α:IL-10 ratio	Higher	Lower
Il4 relative expression.	Higher	Lower
Il6 relative expression	Higher	NS
VCAM-1 expression	NS	Higher

Comparisons were made versus their respective control (infected nondiabetic mice for infected diabetic mice and diabetic nontreated mice for diabetic treated mice).

mice still presented signs of strong ongoing inflammation, characterized by high IFN-γ levels and a high TNF-α:IL-10 ratio, whereas this inflammation was found to be subsided in the nondiabetic groups.

The regulation of genes responsible for inflammatory conditions by insulin has been observed in other studies (23, 24) even in the presence of T1D. We observed higher *il6* expression in T1D mice infected with Pb18 than in noninfected T1D mice, and treatment with insulin seemed to decrease the expression of this gene. Another interesting finding was that i*l4* gene expression was higher in T1D mice infected with Pb18 than in noninfected T1D mice. The progression of PCM is associated with high IL-4 production (40). Although a Th1 immune response appears to effectively control infection, the fungus itself is able to modulate metabolite production and surface molecule interactions with immune cells to produce both pro- and anti-inflammatory cytokines, which makes it difficult for the body to develop resistance during infection (41). In this regard, studies show that an impaired immune response in diabetes facilitates the establishment of infection (22), which may justify the dichotomy observed in the relative gene expression of both pro- and anti-inflammatory cytokines but may not reflect the secreted protein scenario. To better understand the effect of insulin on the inflammatory focus, we analyzed signaling pathways in lung homogenate. Proteins in the mitogen-activated protein kinase (MAPK) signaling pathway have important roles in the activation and differentiation of T lymphocytes (42). Studies suggest that ERK phosphorylation is related to T cell polarization to a Th2 profile (43), and p38 phosphorylation is associated with Th1 polarization (44). Viardot and associates showed in 2007 that insulin promoted the differentiation of T cells to a Th2 profile, reducing the Th1 cell proportion and the IFN-γ:IL-4 ratio. These results were associated with increased ERK phosphorylation and reduced p38 phosphorylation (45). In this study, the results observed in the lungs of mice 45 days after Pb18 infection showed that insulin treatment resulted in diminished phosphorylation of ERK in nondiabetic mice, and p-p38 was unaltered. Other results, such as increased IL-4 expression and reduced IFN-γ in this group, oppose a diminished response of the Th2 profile.

The importance of cell migration to the inflammatory site, thus allowing the development of an efficient immunological response, has been evidenced by previous studies showing the role of proinflammatory cytokines and adhesion molecules (6, 46, 47). Studies also show that cell migration through the endothelium is dysregulated in T1D patients, and this is often related to alterations on the expressions of adhesion molecules. Sharma et al. (48) observed that T1D patients with retinopathy had accentuated levels of ICAM-1 compared to controls, and diabetic patients with chronic kidney disease showed increased VCAM-1, both suggesting more severe inflammation in patients with pathologies related to diabetes (48, 49). On the other hand, in animal models of T1D, diabetic rats showed reduced inflammation in addition to lower levels of ICAM-1 compared to nondiabetic rats in response to external inflammatory stimuli (6). In this work, different than infected nondiabetic controls, infected diabetic mice did not show higher expressions of VCAM-1 in the lungs, and although we did not perform a cell migration assay, both the expressions of VCAM-1 in pulmonary vessels and the number of leukocytes in this organ were restored after treatment with insulin. Interestingly, this augmentation of leukocyte numbers was found accompanied by a reduction of IFN-γ levels and TNF-α:IL-10 ratio, suggesting a reduced Th1 inflammation and perhaps involvement of other groups of cytokines that were not addressed by this study. Other than IFN-γ and TNF-α, the presence of IL-6 and IL-23 have been associated to formation and maturation of granulomas (50). Moreover, in their work *in vitro*, Calich and Kashino showed in 1998 that secretions of IL-5 by lymphocytes of mice was associated to susceptibility to PCM when it happened during both earlier and later stages of infection and to resistance when observed only during the later stages of it (51), suggesting that the priming of leukocytes to a Th2-type profile increases susceptibility only when it occurs on early stages of PCM, and that may be more important than the population of leukocytes in the inflammation site, the polarization of the type of cytokines produced by them have direct impact on resistance/susceptibility to PCM.

The clinical relevance of T1D, its increasing incidence worldwide and its impact on health care and on social and economic fields are widely known, and studies to better elucidate its deleterious effects and indirect consequences are important. In conclusion, our work showed an impaired acute response to Pb18 in mice with alloxan-induced diabetes characterized by reduced IFN-γ and IL-12 p70 levels, decreased phagocytic activity, and chronic inflammation 45 days after the infection. Moreover, we suggest that insulin modulates this response, as it restored IL-12 p70 levels during acute inflammation and increased presence of leukocytes in inflammatory sites, leading to a reduction in chronic inflammation in T1D mice.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Brazilian National Council for Control of Animal Experimentation (CONCEA) of the School of Pharmaceutical Sciences (FCF), in the University of São Paulo (USP) (the project is registered under the permit CEUA/FCF/512).

AUTHOR CONTRIBUTIONS

FC and JM elaborated the project and conceived the experiments. FC, SF, FT, LR, JG, and ES conducted the experiments. JM, SA, and SR contributed with reagents, materials, analysis, and expertise. FC and JM wrote this paper and all authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Precision Nutrition in Chronic Inflammation

*Tobias J. Demetrowitsch[1], Kristina Schlicht[2], Carina Knappe[2], Johannes Zimmermann[3], Julia Jensen-Kroll[1], Alina Pisarevskaja[1,4], Fynn Brix[1], Juliane Brandes[2], Corinna Geisler[2], Georgios Marinos[3], Felix Sommer[5], Dominik M. Schulte[2], Christoph Kaleta[3], Vibeke Andersen[6,7,8], Matthias Laudes[2], Karin Schwarz[1] and Silvio Waschina[4]**

[1] Division of Food Technology, Institute of Human Nutrition and Food Science, Kiel University, Kiel, Germany, [2] Division of Endocrinology, Diabetes and Clinical Nutrition, Department of Medicine 1, Kiel University, Kiel, Germany, [3] Research Group Medical Systems Biology, Institute of Experimental Medicine, Kiel University, Kiel, Germany, [4] Division of Nutriinformatics, Institute of Human Nutrition and Food Science, Kiel University, Kiel, Germany, [5] Institute of Clinical Molecular Biology (IKMB), Kiel University, Kiel, Germany, [6] Institute of Regional Research, University of Southern Denmark, Odense, Denmark, [7] Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark, [8] Focused Research Unit for Molecular Diagnostic and Clinical Research, University Hospital of Southern Denmark, Aabenraa, Denmark

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Edited by:
Ralf J. Ludwig,
University of Lübeck, Germany

Reviewed by:
Jean-Pierre Routy,
McGill University, Canada
Artem Vorobyev,
University Medical Center Schleswig-Holstein, Germany

***Correspondence:**
Silvio Waschina
s.waschina@nutrinf.uni-kiel.de

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The molecular foundation of chronic inflammatory diseases (CIDs) can differ markedly between individuals. As our understanding of the biochemical mechanisms underlying individual disease manifestations and progressions expands, new strategies to adjust treatments to the patient's characteristics will continue to profoundly transform clinical practice. Nutrition has long been recognized as an important determinant of inflammatory disease phenotypes and treatment response. Yet empirical work demonstrating the therapeutic effectiveness of patient-tailored nutrition remains scarce. This is mainly due to the challenges presented by long-term effects of nutrition, variations in inter-individual gastrointestinal microbiota, the multiplicity of human metabolic pathways potentially affected by food ingredients, nutrition behavior, and the complexity of food composition. Historically, these challenges have been addressed in both human studies and experimental model laboratory studies primarily by using individual nutrition data collection in tandem with large-scale biomolecular data acquisition (e.g. genomics, metabolomics, etc.). This review highlights recent findings in the field of precision nutrition and their potential implications for the development of personalized treatment strategies for CIDs. It emphasizes the importance of computational approaches to integrate nutritional information into multi-omics data analysis and to predict which molecular mechanisms may explain how nutrients intersect with disease pathways. We conclude that recent findings point towards the unexhausted potential of nutrition as part of personalized medicine in chronic inflammation.

Keywords: nutrition, personalized medicine, inflammation, dietary intervention, disease prevention, microbiota, precision nutrition, chronic diseases

INTRODUCTION

Over the past several decades, increased incidence rates of diseases associated with chronic inflammation, including inflammatory bowel disease (IBD), diabetes, and asthma have been observed in countries experiencing industrial and urban growth (1–3). While the causes of this incidence surge are still highly debated in biology and medicine communities, there is increasing epidemiological evidence that the rise of chronic inflammatory diseases (CIDs) can be attributed to nutritional changes (4–7). A dietary basis for CIDs is further supported by the fact that they frequently involve physiological changes in the gastrointestinal tract including alterations in gut microbiota composition and metabolism (8–11). In this context, a number of studies have identified molecular mechanisms by which dietary components can interact with immunological pathways either directly (12, 13) or indirectly, *via* modulation of the gut microbiota (14, 15).

Patients with the same CID can differ markedly in their precise disease manifestation with respect to inflammation relapse, remission, and response to therapy (16, 17). Studies using clinical cohorts have revealed several molecular features that are associated with disease heterogeneity. These include genetic (18, 19) microbial (20), and metabolic factors (10, 21). The appreciation of the wide range of individual factors influencing the pathology of CIDs intensified research endeavours to further tailor treatment strategies to the patient's molecular characteristics (22, 23).

Given the multitude of molecular mechanisms by which nutrition can intersect with immunological pathways, microbiome dynamics, and human metabolism, nutrition therapy has been recognized as integral to the development of novel personalized CID prevention and disease management strategies (24). Additionally, nutrition has vast potential to contribute to personalized medicine in two ways: first, the patient's nutritional status and dietary intake information can be used to inform new prescriptive biomarkers, i.e. biomarkers that can predict the patient's response to potential treatment strategies (25). Second, nutritional interventions display promise for patient-centered treatments of CIDs.

Those ideas are mirrored in an increasing number of CID-related articles in the scientific literature that also involve aspects of nutrition and diet (**Figure 1**). Yet, empirical studies reporting clear evidence of the effectiveness of using nutrition-derived biomarkers and nutritional interventions in CID therapies are rare, thereby limiting nutrition data-assisted decision making and dietary interventions in clinical practice of personalized medicine. For instance, a recent systematic review combined with an expert survey to derive guidelines for clinical nutrition management in IBD yielded only 7 evidence-based dietary/nutrition recommendations that relate the patient's individual characteristics, e.g. age, current and previous treatments, and nutritional status (26).

The large discrepancy between the anticipated role and actual application of nutrition in personalized CID management is largely due to the intrinsic biochemical complexity of nutrition, including its long-term effects and interaction with various

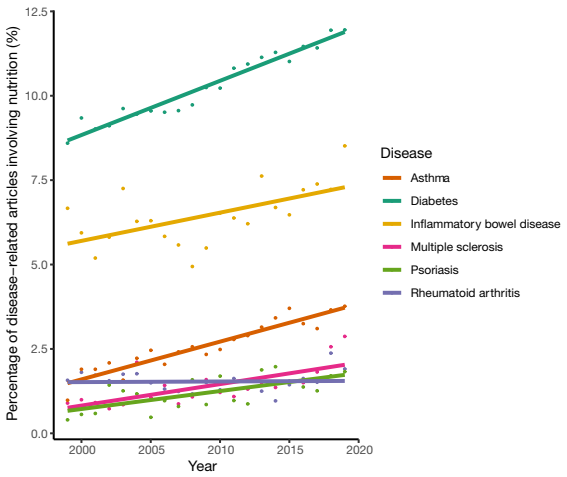

FIGURE 1 | Percentage of CID-related articles, whose titles or abstracts involve the terms "nutrition' or "diet'. The search was performed on July 6th 2020 using PubMed® and limited to the years 1999–2019. Lines denote the results from linear regression analysis. Percentages increased significantly for all diseases (Pearson's product-moment correlation, $P < 0.005$) except for rheumatoid arthritis ($P = 0.87$).

environmental factors (27, 28). However, recent studies have started to address this issue by investigating the impact of nutrients on host organisms alongside the molecular interactions between nutrients, microorganisms, drugs, and host genetics (29). In this review, we highlight recent developments at the interface between nutrition and precision medicine in chronic inflammation. In addition, key challenges in the field are discussed and potential solutions proposed.

NUTRITION AND PRESCRIPTIVE BIOMARKERS

Nutrition is an important determinant of CID patient heterogeneity (30). Thus, specific information about an individual's nutritional status and dietary habits can support data-driven decision making to optimize a patient-tailored treatment. There are a few nutrition-derived objective indicators that predict therapeutic outcomes and are used in clinical practice to adjust CID treatments. For instance, Crohn's Diease patients experiencing extended nutritional deprivation are at increased risk of refeeding syndrome; European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines recommend nutritional supplementation of phosphate and thiamine in such cases (26). In addition, it is well-documented that malnutrition promotes increased risk of morbidity and mortality following surgery in IBD patients (31, 32). Hence, if malnourishment is documented, nutritional support following emergency surgery is commonly recommended (26).

One ambitious goal of precision medicine is to emhance the amount and accuracy of data that describe a patient's nutritional

status, in order to identify novel biomarkers that can predict the clinical outcome of possible treatments. To achieve this goal, nutritional status data should be analyzed in close combination with other personal data as the effects of nutrients are moderated by other influencing factors such as host genetics, body composition or the intestinal microbiome (33). In a seminal study by Zeevi et al. (34), the authors collected longitudinal data from 800 participants including blood glucose levels, microbiome structure and function, nutritional status, and dietary behavior. On the basis of these data, a machine-learning algorithm was devised that was able to predict individual postprandial glycaemic response to a given meal Zeevi et al. (34). This example illustrates that the potential of nutritional information for identifying biomarkers for individual metabolic responses can be substantially potentiated when coupled with additional personal data, such as that related to the intestinal microbiome. Although the construction of such mathematical predictors relies on large cohorts and longitudinal data acquisition, similar approaches may also be applied to clinical CID patient cohorts, where nutritional data is incorporated in the predictor. Such predictors could assists clinicians in making personalized treatment decisions.

Moreover, the possibility of deriving prescriptive biomarkers from nutritional data will further increase as more molecular mechanisms describing the interplay among immunological pathways, the microbiome, and nutrients are elucidated. A series of studies within the past decade provided new evidence that the microbiome could act as a crucial intermediary between diet and inflammatory diseases (35). This complex relationship between dietary compounds, the microbiome, and inflammation is probably best elucidated for the anti-inflammatory effects of microbiome-derived butyrate. Butyrate and other short-chain fatty acids (SCFAs) are produced in large amounts through fermentation of dietary fibres by certain bacterial species (e.g. *Bacteroides fragilis*) that possess the enzymatic machinery to degrade those compounds (36). The diet-dependent and immunomodulating role of butyrate is especially interesting in the light of recent studies that demonstrated an association between colonic butyrate with clinical outcomes of CID therapies. For instance, it has been shown that butyrate levels are associated with clinical remission following anti-TNFα therapy in IBD patients (10, 37). Similarly, the efficacy of anti-CTLA-4 immunotherapy has been reported to be associated with the proportion of specific butyrate-producing bacteria within melanoma patients' gut microbiota (38, 39). Moreover, a reduction in colonic butyrate-producing bacteria has been reported in HIV infection (40) and appears to affect the response to antiretroviral therapy (41). Such studies emphasise the potential to combine dietary information and microbiome data to approximate intestinal butyrate production capacity and, hence, individual responses to therapies.

Several molecular mechanisms have been elucidated how butyrate interacts with immunological pathways, illustrating the compound's central role in CIDs. Smith et al. (14) have identified specialized butyrate-sensing receptors expressed by anti-inflammatory regulatory T-cells, whose differentiation is stimulated by butyrate (42). Butyrate also functions as a potent inhibitor of histone deacetylase enzymes, thereby linking microbial metabolites to the regulation of host transcriptional profiles (43, 44). Furthermore, Li et al. (45) have shown in a cell culture model that butyrate activates pyruvate kinase M2, leading to substantially altered cell metabolism, and thereby suppressing the proliferation of colorectal cancer cells.

Besides butyrate and other SCFAs, gastrointestinal micro-organisms transform dietary components to contribute a wide range of additional compounds to the human metabolome (46). Future research on the role of these metabolites in health and disease will yield additional biomarkers and targets for personalized treatment of CIDs.

PERSONALIZED NUTRITIONAL INTERVENTIONS

Nutrition is likely the largest toolbox we have at hand to influence both metabolic processes in the human body and the intestinal microbiome's structure and function. Thus, nutritional interventions represent a promising strategy for personalizing CID treatments. Nevertheless, various generalized nutritional recommendations, established over the past four decades in the context of chronic diseases, have not noticeably diminished their incidence (47).

New targets for nutritional interventions are expected to emerge as our mechanistic understanding of inflammatory diseases and the effect of nutrition on the immune system expands. For example, the above-mentioned anti-inflammatory effect of butyrate and its involvement in maintaining gastrointestinal health has promted researchers to explore using targeted dietary interventions to increase its intestinal production. Marino et al. (35) have shown that administration of a diet yielding high butyrate levels through gut microbial fermentation enhanced gut integrity, increased the number and activity of regulatory T cells, and decelerated the progression of diabetes in a diabetic mouse strain model. Such results exemplify the potential of dietary interventions to specifically target immunomodulating microorganisms. Thus, quantification of patient-specific activity of molecular processes (e.g. *via* coupled metagenomics and metabolomics) associated with the maintenance or initiation of CID remission are highly promising indicators for the efficacy of nutritional interventions targeted to modulate them.

Another intriguing development with implications for nutritional interventions are recent findings that the therapeutic effects of drugs are mediated through complex interactions between the bioactive agent, dietary compounds, and microorganisms (48, 49). These include pharmaceuticals frequently administered to treat certain CIDs, such as sulfasalazine or metformin (49, 50). In the case of metformin, a medication for the treatment of type 2 diabetes, Pryor et al. (50) have shown using a *Caenorhabditis elegans* model system that nutrition influences the animal's response to the drug, and that the effects are mediated by the gut bacterium *Escherichia coli*. Specifically, metformin's impact on host metabolism and lifespan are attributed to an increased production

of agmatine, which further depends on nitrogen-containing compounds in the nematode's diet, namely amino acids, amino sugars, and nucleotides. As part of the same study, reanalysis of microbiome data from four independent human cohorts indicated that the abundance of bacteria capable of producing agmatine increased in conjunction with metformin treatment. This suggests that similar synergistic interactions between the drug and nutrients may occur also in humans. In general, a mechanistic understanding of nutrient-drug interactions could pave the way to personalized CID treatment strategies that combine pharmacological and nutritional interventions.

Nutritional interventions that specifically target colonic microorganisms might be hindered by nutrient absorption in the small intestine. For instance, the vitamin niacin has been shown to beneficially affect intestinal homeostasis and decrease susceptibility to intestinal inflammation in a mouse model system (51). Fangmann et al. (52) employed a food-technological approach to deliver high amounts of nicotinic acid into the colon by micro-encapsulating the compound, thereby delaying its release until the capsules reached the ileocolonic region. *In vivo* administration of the capsules in humans changed the microbiota composition in ways that are commonly considered favourable; i.e. the increased abundance of *Bacteroidetes*. In addition, biomarkers of systemic insulin sensitivity and metabolic inflammation improved without observable negative side-effects (e.g. facial flushing) that have been described for un-capsulated orally administered niacin (52). Thus, food-technological approaches may promote the development of nutritional interventions for precision medicine by increasing the intervention efficacy and reducing unwanted side effects. In addition, several preclinical models demonstrated the potential of microbiome-directed nutritional interventions for the treatment of malnutrition and its associated inflammatory complications (53–55) and promising initial results were recently gained in preliminary clinical studies (56, 57).

CHALLENGES AND POTENTIAL SOLUTIONS

Precision in Dietary Assessment

Technological advances over the past decade have elevated the degree of precision with which a person can be characterized on both the genetic level (i.e. genomics) and phenotypic level (i.e. transcriptomics, proteomics, metabolomics). Available methods for assessing the environmental factor of *nutrition* (in terms of the person's food consumption and habits) do not currently provide the same degree of detail in most cases. An exception is nutrition provided during intensive care, where nutritional intake is usually well documented, e.g. for preterm infants in neonatal intensive care units (58). In most other human cohort studies, nutrition is typically recorded using dietary questionnaires. Such questionnaires have the disadvantage that realiability of the data obtained may be limited, since it is based on the subjective perception of the study participant (59). Several software solutions (mainly mobile apps) have emerged that aim to increase dietary data quality (e.g. *via* incorporating automatic food item recognition from images), but that entail their own data acquisition shortcomings as reviewed elsewhere (60). While some of these solutions are already in use in biomedical research projects [e.g. (61)], a major issue remains: the dietary information obtained cannot be treated as objective and thereby does not meet the criteria for a source of potential medical biomarkers (62).

A promising approach to address this challenge is the identification of novel food intake biomarkers, which are molecularly-based objective indicators derived from human samples (63). The idea is to estimate previous dietary intake by measurements of dietary compounds or derived chemicals in human matrices such as blood, urine, faeces, hair, or dental calculus. Ongoing research focuses on the identification and evaluation of a wide range of different food intake biomarkers using metabolomics techniques (64). If proven applicable, such biomarkers will reveal new links between nutrition and inflammatory disease mechanisms.

Nutrition and the Curse of Dimensionality

Food is molecularly complex. Online Databases such as FooDB (65) or FoodData (66) enable users to approximate the amount of macro- and micronutrients in a given diet. Thus, if personal dietary information is available for a large study cohort involving CID patients, one could statistically test for associations between individual nutrients and the patients' disease manifestation and progression parameters. Yet, as Bauer et al. (27) pointed out, this approach would be hindered by the so-called *curse of dimensionality*, where the number of features (nutrients) quickly becomes larger than the number of samples (individuals), which often makes it difficult to distinguish real differences from differences that occur by chance. The true integration of omics data has been a major task in biomedical research in recent years, and several promising bioinformatics approaches have emerged [see Pinu et al. (67); Huang et al. (68) for reviews].

The basic idea behind multi-omics data analysis is to combine multiple biological features in a single analysis, in order to simulate phenotypic and environmental complexity and interrelatedness of biological systems as close as possible to the true nature of things. According to de Toro-Martín et al. (69), this includes deep phenotyping, physical activity, food behavior, and dietary habits in combination with multi-omics data. In fact, multi-omics data analysis might be a major driving force on the way to personalized medicine; some progress has been made in cancer research in particular (70), though to date none of these tools has enough predictive power for routine clinical use. This might be, because many tools still apply a sequential approach by analyzing one data layer after another and integrating the results post-analysis. As de Anda-Jáuregui and Hernández-Lemus (71) pointed out, biological processes and phenomena are not comprised of single, independent layers of biological features, and therefore algorithms that can simultaneously analyze multiple data types are preferred.

In multi-omics data analysis, an important distinction must be made between candidate/hypothesis-driven methods and more exploratory approaches employing dimensionality

reduction techniques, such as principal component analysis. While the former has a potential drawback of information loss if the full data collected are no incorporated, the latter shows weaknesses in integrating biological system background knowledge. In addition, integration of non-omics data, like clinical phenotypes and nutritional data with omics data layers (e.g. gene variants, transcriptomes and microbiome data) is even more challenging, due to the heterogeneity of data types, possible interactions, and the existence of sub-phenotypes (72). To address those points, we need complex mathematical and bioinformatics methods [see Bersanelli et al. (73)] for a review] and careful attention to the preparation (such as standardization and normalization) and quality control in the different data types. Tools evolving from the field of systems biology, like metabolic network and pathway analysis, are incorporating known interactions between genes, proteins, micronutritional supply, and molecules, and can contribute significantly to the rapidly developing field of multi-omics analysis (74).

Identifying Molecular Mechanisms

A major challenge in the identification of potential nutritional interventions is the elucidation of the molecular mechanisms illustrating how nutrition and specific dietary compounds influence immunological pathways. High-throughput technologies such as metagenomics and metabolomics have been applied to describe quantitative associations of personal nutritional data with metabolism, inflammation, and the microbiome in health and disease (75–77). Yet, these associations usually do not allow conclusions on underlying molecular mechanisms.

To investigate mechanisms, experimental model systems can be used, which provide valuable insights into key aspects of the molecular links between nutrition, immune system response, microbial processes, and inflammation (78). Animal models (e.g. mice, *C. elegans*) or cultures of established cell lines can be applied to elucidate general mechanisms and to screen many different combinations of potential influencing factors (48, 50). However, the translation of such results to human subjects is limited and cannot represent the heterogeneity in immune responses among human individuals. A promising alternative are *ex vivo* and *in vitro* model systems where biomaterial (e.g. tissue, cells, stool) are directly sampled from human individuals. *Ex vivo* and *in vitro* model systems enable large-scale phenotyping experiments under controlled conditions, and make it possible to link results directly with the individuals' unique characteristics. Thus, such model systems are of vast interest in precision medicine. Available and predicted future model systems for human immunology are reviewed in detail Wagar et al. (78).

Computer models of biochemical processes are powerful tools for investigating the effect of nutrition on human metabolism and gut microbial processes. Various modelling methods exists [see Kumar et al. (79) for review], which share the common feature that system elements, namely metabolites, proteins, and genes, are represented in nodes within a network, where edges represent known relationships such as biochemical transformations, gene expression, and regulation. Thus, these network models allow predictions about metabolic flux distributions through metabolic networks in a given nutritional environment including the role of individual genes and proteins (79). The *in silico* simulations are vastly scalable, which enables researchers to perform simulations for a wide range of different scenarios (e.g. diets) as well as potential perturbations. Furthermore, theoretical models can be parametrized based on different data (e.g. abundance of specific proteins, transcripts, microorganisms, diet) from human individuals in order to frame the model to represent the individual's conditions (50, 74). Results obtained from *in silico* models are useful to generate hypotheses about complex molecular mechanisms, which can subsequently be scrutinized by targeted experiments.

DISCUSSION

Clinicians have always striven to provide the best recommendations based on the patient's characteristics and particular disease manifestation (23). Since nutrition is an important factor with vast impact on human health, nutritional interventions are often considered promising components in the treatment of a wide range of diseases. In some diseases, for which the molecular pathophysiology is well-understood, nutritional interventions have been proven to be highly effective, for instance in the treatment of phenylketonuria or coeliac disease (80). It is the ambitious goal of precision nutrition in chronic inflammation to achieve similar success with the help of nutrition-derived biomarkers and personalized nutritional interventions. This is a difficult task since CIDs arise from complex gene-microbiome-environment interactions (2) in which most underlying molecular mechanisms remain obscure. In this review, we discussed recent studies which address this issue and revealed nutrition's vast and unexhausted potential in the treatment of CIDs by elucidating its impact on disease-related molecular pathways. Based on the applied methodologies in the reviewed studies and the current challenges discussed, we emphasize that future research in the field of nutrition in precision medicine for CIDs should focus on: (i) obtaining detailed nutritional data alongside omics-data (i.e. genomics, metagenomics, metabolomics) in human cohort studies in clinical contexts as well for population-level cohorts; (ii) development of novel mathematical methods to integrate different data sources in a systems biology framework that represents the relationship between measured molecular features; and (iii) elucidating molecular mechanisms describing how nutrition affects immunological pathways, including the modulating effects of drugs and the intestinal microbiota.

AUTHOR CONTRIBUTIONS

ML, KSchw, and SW conceptualized the review manuscript. SW took lead in writing the manuscript with contributions by TD, KSchl, KSchw, CKn, and JZ. All authors contributed to the article and approved the submitted version.

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Phenotyping of Adaptive Immune Responses in Inflammatory Diseases

Jens Y. Humrich[1], Joana P. Bernardes[2], Ralf J. Ludwig[3], David Klatzmann[4] and Alexander Scheffold[5]*

[1] Department of Rheumatology and Clinical Immunology, University Hospital Schleswig-Holstein—Campus Lübeck, Lübeck, Germany, [2] Institute of Clinical Molecular Biology, Christian-Albrechts-University Kiel, Kiel, Germany, [3] Lübeck Institute of Experimental Dermatology (LIED), University of Lübeck, Lübeck, Germany, [4] Sorbonne Université, INSERM, Immunology-Immunopathology-Immunotherapy (i3), Paris, France, [5] Institute of Immunology, Christian-Albrechts-University Kiel, Kiel, Germany

Immunophenotyping on the molecular and cellular level is a central aspect for characterization of patients with inflammatory diseases, both to better understand disease etiopathogenesis and based on this to develop diagnostic and prognostic biomarkers which allow patient stratification and tailor-made treatment strategies. Technology-driven developments have considerably expanded the range of analysis tools. Especially the analysis of adaptive immune responses, often regarded as central though mostly poorly characterized disease drivers, is a major focus of personalized medicine. The identification of the disease-relevant antigens and characterization of corresponding antigen-specific lymphocytes in individual patients benefits significantly from recent developments in cytometry by sequencing and proteomics. The aim of this workshop was to identify the important developments for state-of-the-art immunophenotyping for clinical application and precision medicine. We focused here on recent key developments in analysis of antigen-specific lymphocytes, sequencing, and proteomics approaches, their relevance in precision medicine and the discussion of the major challenges and opportunities for the future.

Keywords: immunophenotyping, sequencing, proteomics, inflammation, precision medicine, TCR repertoire, antigens

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****Correspondence:***
Jens Y. Humrich
jens.humrich@uksh.de

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INTRODUCTION

Immunophenotyping is a key method for monitoring and diagnosing of immunological diseases, such as infectious diseases, chronic inflammatory and autoimmune diseases and allergy. For many of these the actual disease cause is still elusive or as in the case of COVID-19 the origin and contribution of potentially pathogenic immune reactions to severe disease. Classically immune monitoring is accomplished *via* measurement of serum biomarkers, including acute phase proteins, serum cytokines, or antigen-specific serum antibodies. However, a deeper understanding of the underlying disease cause and the pathogenic mechanisms, requires the identification and characterization of the responsible immune cells and their interaction partners. Most of these

diseases are restricted to specific organs or antigens and there is plenty of, though mostly indirect, evidence that antigen-specific lymphocytes are the pathogenic drivers of disease. However, due to technological restrictions these cells are barely characterized and in many inflammatory and autoimmune diseases the target antigens of the pathogenic immune reaction are still unknown. This is a major roadblock for development of personalized medicine, that is disease- and patient-specific diagnostics and therapies targeting the disease cause rather than the symptoms.

Thus a major aim of immunophenotyping in the age of personalized medicine, diagnostics and therapy is the development of tools allowing to dissect the immune response on the level of antigen-specific T and B cells, their relevant interaction partners and effector molecules. During a workshop on immunophenotyping in inflammatory diseases, which took place at the international symposium of the Cluster of Excellence "Precision Medicine in Chronic Inflammation (PMI)" of the universities of Kiel and Lübeck on February 18th 2020 in Hamburg, Germany, recent developments facilitating such antigen-specific approaches and beyond were discussed.

STATE-OF-THE-ART AND KEY DEVELOPMENTS

Flow-Cytometry and Analysis of Antigen-Specific Lymphocytes

The method of choice for detailed characterization of immune cell composition and phenotype is flow-cytometry, which does not only allow rapid quantitation of the main leukocyte subsets but also their detailed phenotypic characterization, including the differentiation of the complex cosmos of lymphocyte subpopulations at a single cell level. A state-of-art overview of currently available cytometric technologies can be found under (1). Instrumental to this has been the steadily increasing numbers of parameters, which can be measured from individual cells. Theoretically > 40 parameters per cell are currently possible, although this is still not part of the clinical routine. However, recent developments especially of multi-laser instruments or spectral cytometers have significantly improved the ease of use, especially for the combination of various fluorescent dyes within multiplex panels (1–4). This may bring multi-parameter flow-cytometry closer to the clinical diagnostic lab reality.

Identification of antigen-specific lymphocytes is a challenge due to their low frequency and the availability of antigen-reagents allowing direct labeling of their antigen-receptor (5, 6). Although fluorescently tagged antigens can in principle be used to directly label B cells these are still no routine reagents and staining procedures (1). Even more challenging the exact peptide/MHC combinations need to be defined to generate the recombinant peptide/MHC-multimers for antigen-specific T cell staining. This is a significant difficulty especially for the analysis of human T cells due to the high HLA-diversity in general and in particular for CD4+ T cells due to low quality MHC class II reagents, complex antigens or pathogens and/or unknown peptide targets. Therefore especially human CD4+ T cell analysis is mainly based on functional read-outs, following *in vitro* stimulation with antigenic peptides, proteins or protein extracts. Several technologies to detect antigen-reactive T cells have been developed (1, 5, 6). A common problem for all antigen-specific assays is the low frequency of target cells. A frequent solution to this is the *in vitro* expansion of rare T and B cells prior to analysis. However, this introduces an unpredictable bias with regard to phenotype, function and selection of certain cell subsets. Direct approaches utilizing minimal stimulation times (5–7 h) combined with powerful rare cells enrichment strategies, e.g., *via* magnetic cell separation, provide a more direct view (7–9). Rapid induction of CD154 expression in all conventional T helper cells (Tcon) (10, 11) and of CD137 in Tregs (12) within a short time window of 5–7 h after *in vitro* antigen stimulation has proven to be highly sensitive and specific to identify essentially all reactive CD4+ T cells. This can be combined with magnetic enrichment strategies, termed antigen-specific T cell enrichment (ARTE) (7). This technology has been used to characterize human CD4+ T cell responses in a number of clinical settings, including allergy (13), IBD (13, 14) and infectious diseases (15). The unique strength of direct enrichment-based approaches is the access to highly purified antigen-specific T cells, e.g., *via* fluorescence-based flow-sorting technologies, which is ideal for combination with downstream "-omics" technologies, allowing their deep molecular profiling as well as functional analyses in unparalleled resolution (13, 14, 16) (**Figures 1B, C**). This includes TCR sequencing to determine T cell clonality and/or TCR affinity measurements (13, 14, 16) (**Figures 1A, C**). The particular relevance of activation-based assays has been highlighted by recent data on SARS-CoV-2-specific T cells which allowed rapid identification of target proteins and overall T cell reactivity in patients (17–19) as well as clonality and affinity differences between mild and severe COVID-19 (20). In general B/TCR sequencing provides unique insight in B and T cell composition as outlined below. However, it has to be emphasized here, that the combination of antigen-specific B/T cell sorting with antigen-receptor sequencing is particularly powerful. Antigen-receptor sequences from cells with known specificity can be used as molecular identifiers to track antigen-specific clones in samples from the same patient. This has been used, for example, to track the modulation of antigen-specific T cells in bulk TCR sequencing data from patients with multiple sclerosis during pregnancy (21). Similarly, known TCR sequences from paired blood and tissue samples will allow identification of antigen-specific T cells from bulk or single cell RNA sequencing data. The latter approach may allow to follow and deeply characterize antigen-specific T cells even in small tissue samples.

TCR Repertoire Analysis

Clonal expansion is one of the hallmarks of adaptive immune responses. Therefore T cell receptor (TCR) analyses carry great potential for investigating the specificity of the T cell immune response under physiological and pathophysiological conditions. There are however multiple issues to consider in advance. (i) The first is about numbers. As outlined above antigen-specific lymphocytes are rare. From the possible 10^{19} theoretically possible combinations (22), TCR selection during thymopoiesis selects for each individual a repertoire of approximately 10^{7} to 10^{8} unique TCRs (23). Thus looking for specific TCRs, even expanded

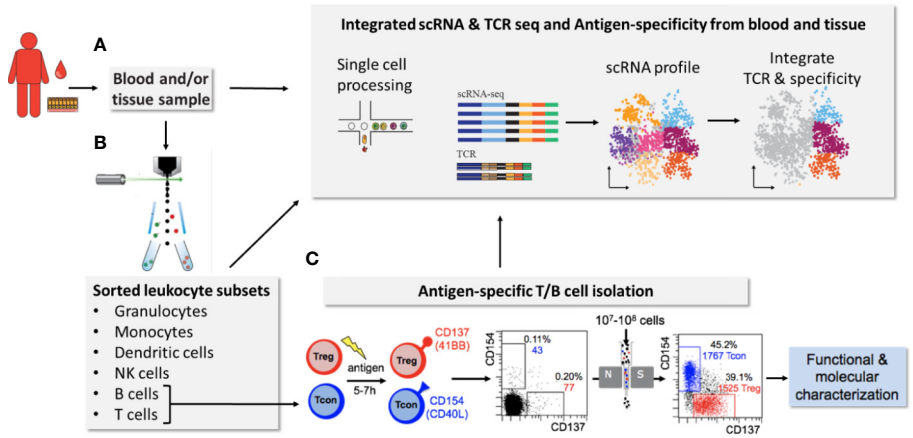

FIGURE 1 | Integrated scRNA & TCR seq and antigen-specificity from blood and tissue. (Single cell) RNAseq provides unique multi-parameter information of single cells **(A)** or sorted cellular subsets **(B)**. Further the combination of antigen-receptor sequencing with scRNASeq allows correlation of RNA expression with clonal lymphocyte populations **(A)**. In addition, antigen-receptor sequences derived from antigen-specific lymphocytes of the same donor, obtained *via* combined magnetic pre-enrichment and fluorescence based flow-sorting, allows to integrate antigen-specificity and certain functional aspects **(C)**. TCR/BCR information from antigen-specific lymphocytes may also be used to track antigen-specific cells within small tissue samples.

ones, resembles searching for a needle in a haystack. (ii) The second is about sampling: where, and how much? Indeed, how much of the immune response targeting a pulmonary infection is represented in 10^5 cells from blood? (iii) The third is about T cell functional diversity. As an example, since Treg and effector T cells have opposite effects, what would mean the identification of a given TCR if you cannot assign it to one or the other cell population? (iv) The fourth is about technology: TCRs are made of alpha and beta chains that cannot (accurately) be paired by knowledge. Only single-cell sequencing can provide paired chains. Also, the accuracy of the technology that heavily relies on PCR amplification and its capacity to detect rare clonotypes might affect results (24). Despite all these limitations, TCR sequencing carries enormous potential in particular in combination with cytometric techniques discussed above. There are currently two major approaches to TCR studies: (i) global TCR repertoire assessment from a large number of cells, classically applied to study peripheral blood T cells, and which can generate millions of alpha and beta unpaired TCRs per sample, and (ii) single-cell sequencing of smaller samples, such as tissue infiltrating cells, which generates in the order of 10^3-10^4 clonotypes. Global TCR studies are providing much larger repertoires, from which the challenge is to identify the relevant TCRs. Numerous algorithms have been and are still developed to extract relevant information, such as repertoire diversity, expansions of rare cells and cross-reactivity (25–30). They, for example, allowed to identify the TCRs responding to vaccination (31) and in our experiments identified TCRs are specific and even predictive of given autoimmune diseases (David Klatzmann, unpublished observations). In contrast, single-cell studies have the advantages to relate TCRs directly to a setting/tissue or an ongoing immune response and to provide their paired alpha and beta chains (32). Noteworthy, it also allows to concomitantly investigate, (i) the binding of dextramers or MHC-multimers to give clues on the cell specificity, and (ii) the single-cell transcriptome to provide, for example, the activation status of cells with specific TCRs (32, 33).

Bulk and single cell TCR sequencing are truly complementary and even synergistic. Bulk TCR sequencing allows to provide general information on the repertoire, with indices reflecting its diversity and "normality" (25–30). Also, appropriate algorithms can identify thousands of TCRs of potential interest, based for example on their frequency in the sample compared to their theoretical probability of generation (34). The relevance of these sequences can further be explored by blasting them with databases of TCRs with known specificities (35, 36). On the other hand, single-cell sequencing, despite its limitation in terms of the number of sequences, has the advantage of providing the paired sequences in link to a specific setting. For example, the T cells present in bronchoalveolar lavages of patients with lung infection (33) or the cerebro-spinal fluid of patients with neurogenerative diseases can reasonably be assumed to be part of the ongoing immune reaction associated with the condition. Thus, as a recommendation, we would suggest the following:

- To sequence the bulk repertoire of at least 10^5 purified/enriched T cell populations, at least always separating Treg from Teffs, and using a robust method (24).
- To refrain from performing single-cell TCR sequencing from peripheral blood except if relevant subsets can be enriched, i.e., by antigen-specific cytometry assays as described above.
- To perform single-cell TCR sequencing on samples originating from a small cell population, whether this comes from their location or their expression of relevant markers.

Besides, one should make sure to analyze the obtained datasets with experts of the field and not forget to make their datasets public such as not to waste precious and costly results (37). Indeed, even if we could analyze the entire TCR repertoire

of billions of humans bearing each 10^{12} T cells, we would still be far from approaching the 10^{61} different sequences that theoretically could be produced by the T cell machinery (38). Thus, any TCR sequence that was actually produced (i.e., detected in a sample) had dramatically much smaller chances to appear that the reader has to win the lottery, and thus represents extremely valuable knowledge (from theory to reality) that must be memorized.

RNA Sequencing

High-throughput sequencing methods have been the key to disentangle immune phenotypes of inflammatory diseases for decades. Advances in mapping disease activity, identification of early disease, and therapy-related molecular markers have been made possible by the widespread methodology of bulk RNA-seq, a robust and nowadays inexpensive method of sequencing the transcriptome. Despite the effort, the current number of direct applications of molecular-based disease diagnostic and treatment remains limited (39). One explanation for the poor output of bulk RNA-seq-driven methods is that it solely depicts the transcriptome of a homogeneous and unidentifiable agglomerate of different cell types. Thus, masking cell-specific transcription changes and neglecting the impact of cell type proportions in diseased tissues. Moreover, skewed levels of expression of molecular markers are expected when researchers focus on the immune compartment and particular aberrant proportions in inflamed tissues (40–42).

A possible approach to overcome the lack of cell-specificity is the implementation of single-cell sequencing (scRNA-seq), which generates a transcriptional landscape by cell type. This methodology is capable of acquiring up to 80,000 cells per run, enabling unprecedented power to study patterns of expression of even rare cell types linked to inflammatory diseases (43). Moreover, researchers can customize cell type identification based on their need, by using cell-specific signature genes (e.g., *MKI67* high expression for proliferative lymphocytes) coupled with numerous reference transcriptomic datasets (44). Recent technological advances have also provided access to new layers of information by merging cell-specific expression with TCR or BCR information (45, 46) and one can even produce large-scale immunophenotyping panels of tens to hundreds of antibodies (47). This type of analysis leads to the unbiased classification of T or B cell populations of interest and the relationship TCR or BCR with the respectively cell-specific transcription. However, this methodology not only comes with a very high price tag but also with its own set of challenges [for review please see (48)]: (*i*) scRNA-seq only represents a subset of tissue, thus rare cell-types, such as antigen-specific lymphocytes might be missed by chance if the number of cells acquired is not suitable (49). (*ii*) Experimentally, the methods are prone to batch effects, e.g., time from acquiring sample to sample preparation is inversely correlated with sample quality and stability of expression data (50), different technologies and protocols may also acquire different cell proportions and gene counts (51). (*iii*) Despite the robust methods available for PBMCs, dissociation of fragile tissues such as the brain or intestinal epithelium, might be problematic and carry on skewed assessments of the tissue (52). (*iv*) Specific to TCR applications, the reads might be aligned to an incomplete "reference" genome that might also be biased by somatic rearrangements and mutations (45).

Regardless of the methodological pitfalls, crucial advances in immune profiling and particularly in mapping inflammatory diseases have been pinned to scRNA-seq technology: by integrating scRNA-seq and antigen-receptor sequencing (**Figure 1A**), Boland et al. have contributed to resolving clonal relationships of the PBMCs and the intestinal mucosa in ulcerative colitis by coupling an enrichment for IgG1+ in plasma cells in the inflamed tissue with a specific γδ T cell subset in the peripheral blood of patients (53). This publication elegantly highlights how combining gene expression data paired with receptor sequence enables re-expression and testing for antigen binding and function of potentially relevant molecular markers. Taken all together, scRNA-seq has quickly become the go-to technology for immune profiling of individual cells on a large scale.

Proteomics Approaches

In addition to multicolor flow-cytometry and transcriptomics, proteomics of sorted cell subsets and various tissues allow to provide detailed insights into pathology-associated changes in complex immune-mediated diseases (54). There are a multitude of proteomic techniques that can be used to analyze changes of protein expression; e.g., mass spectrometry-based proteomics (55, 56). This, however, requires a high degree of specialization and access to respective core facilities. Thus, we here focus on proteomic techniques that can, in principle, be applied in most laboratories: (i) Bead-based multiplex arrays for flow cytometry to determine cytokine concentrations, (ii) protein arrays for autoantibody profiling, (iii) proximity extension assay proteomics, (iv) multi-parameter immunohistochemistry, and (v) determination of kinase activity.

Bead-based multiplex arrays for flow cytometry have become a standard technology for the fast and high-throughput analysis of cytokine concentrations in biological samples, such as blood or cell culture supernatants (57, 58).

Several companies offer auto-antigen protein arrays that allow the simultaneous detection of multiple autoantibodies from human serum samples, including the immunoglobulin subtype differentiation (59). These led to the identification of autoantibodies in diseases that are currently not considered as autoimmune diseases, e.g., chronic obstructive pulmonary disease (60) or neuropsychiatric diseases (61). Furthermore, autoantibody arrays can be used to identify additional autoantibody reactivity beyond known autoantibodies, for example in pemphigus (62, 63). However, the mere detection of autoantibodies in any given population is usually not informative because autoantibodies can be detected at high frequency also in healthy blood donors (64, 65) and, are even present in IVIG preparations (66) used to treat autoimmune diseases (67). Thus, in depth characterization of their functions and of the corresponding B and T cells is mandatory.

Proximity extension assay (PEA) proteomics allow to identify up to 1500 human proteins in any solution. The assay is optimized for serum, but use of lysed tissue extracts has also been described (68, 69). For PEA, samples are

incubated with a pair of oligonucleotide-labeled antibodies binding to different epitopes of a specific protein. When these antibodies are in close proximity, they hybridize, and are extended by a DNA polymerase. This unique DNA barcode is then amplified by PCR, whereby the amount of each specific DNA barcode in quantified by PCR (70). Regarding mouse samples, a limited number of proteins (90–100) can be analyzed using this technique. However, up to now, the majority of published reports on PEA have investigated patients with chronic inflammatory skin conditions, but not with systemic inflammatory or autoimmune diseases (69, 71–74). Based on our own experience, we believe that the PEA technology will increasingly be used to study alterations of proteomic signatures in chronic inflammatory diseases, and thus will significantly contribute to the understanding of disease pathogenesis. PEA also offers the possibility to stratify certain patients according to their proteomic signature, and/or allow to predict treatment responses.

While all of the above technologies are primarily used to identify proteomic signatures in blood, multi-parameter immunohistochemistry enables to determine protein expression in tissues. One platform is the multi-epitope ligand cartography (MELC) robot technology that has been described in 2006 (75). The principle behind MELC is repeated staining and subsequent bleaching of histology specimens with automated image recording. Interestingly, only few papers have been published using this technique. Overall, we believe that multi-parameter immunohistochemistry is a potentially powerful tool to understand both protein expression and location, however, may not be suitable for routine diagnostics due to high costs and low sample throughput.

Aberrant kinase activation, in particular of the Janus Kinase (JAK) family, has been identified as a therapeutic target for an increasing number of autoimmune diseases (76–78). A relatively novel method to determine kinase activity in cell culture, as well as tissue or blood samples, is a peptide microarray, commercialized under the name of PamGene (79). In this assay, either serine or threonine-containing peptides are spotted on a microarray. After application of the cell or tissue homogenates, kinases within these phosphorylate their respective substrates. Substrate phosphorylation is detected over time using specific antibodies. Based on the phosphorylation pattern of substrates, the activity of kinases can be determined. This peptide microarray for detection of kinase activity can, among others, be used for the identification of novel therapeutic targets (80, 81), discovery of biomarkers (82, 83), and understanding of physiological immune functions (84). The main focus of the PamGene has so far, however, been in the field of cancer research. Based on our own experience with this technique, we expect that this microarray technique will significantly contribute to our understanding of the pathogenesis of chronic inflammatory and autoimmune diseases in the near future.

PERSPECTIVE

Despite significant progress in translational and personalized medicine in the last decade, there is still an urgent need to identify disease-specific signatures and individual biomarkers allowing patient stratification, prediction of responses to therapy including the assessment of dynamic changes and the identification of novel therapeutic targets. In addition, it should be mentioned that data validation and knowledge transfer into clinical routine is often missing in the majority of applied approaches. Modern high-throughput technologies can be extremely useful and time saving in immunological research and become increasingly relevant especially for precision medicine, but are also highly cost intensive. Thus, the right method or combination of methods suitable to adequately address the questions of your study needs to be carefully selected to get a reasonable balance between costs and benefits. On the other hand one has to consider in advance which relevant information and conclusions can be extracted from multi-parameter data sets and how these data can be combined and integrated in a biological and clinical context. The involvement of experienced system biologist and biostatisticians in the planning of a study is therefore highly recommended in order to get most information from the applied methods.

AUTHOR CONTRIBUTIONS

JH, JB, RL, DK, and AS equally contributed to this review according to their area of expertise and with regard to content and structure of the manuscript. All authors contributed to the article and approved the submitted version

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What Makes Antibodies Against G Protein-Coupled Receptors so Special? A Novel Concept to Understand Chronic Diseases

Gabriela Riemekasten[1,2,3,4]*, *Frank Petersen*[2,3,4] *and Harald Heidecke*[5]

[1] Clinic of Rheumatology and Clinical Immunology, University Hospital Schleswig-Holstein, University of Lübeck, Lübeck, Germany, [2] Research Center Borstel, Division of Pulmonary Immune Diseases, Member of the German Center for Lung Research (DZL), Borstel, Germany, [3] Research Center Borstel, Member of the German Center for Lung Research (DZL), Borstel, Germany, [4] Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Borstel, Germany, [5] CellTrend GmbH, Luckenwalde, Germany

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Fulvio D'Acquisto,
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Rauf Latif,
Icahn School of Medicine at Mount Sinai, United States

***Correspondence:**
Gabriela Riemekasten
gabriela.riemekasten@uksh.de

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Expressions of G protein-coupled receptors (GPCR) on immune and tissue resident cells are the consequence of the cellular environment, which is highly variable. As discussed here, antibodies directed to GPCR (GPCR abs), their levels and correlations to other abs, serve as biomarkers for various diseases. They also could reflect the individual interplay between the environment and the immune system. Thus, GPCR abs could display pathogenic chronic conditions and could help to identify disease-related pathways. Moreover, by acting as ligands to their corresponding receptors, GPCR abs modulate autoimmune as well as non-autoimmune diseases. This article introduces GPCR abs as drivers for diseases by their capability to induce a specific signaling and by determining immune cell homeostasis. The identification of the individual GPCR ab function is challenging but might be pivotal in the comprehension of the aetiology of diseases. This, hopefully, will lead to the identification of novel therapeutic strategies. This article provides an overview about concepts and recent developments in research. Accordingly, GPCR abs could represent ideal candidates for precision medicine. Here, we introduce the term antibodiom to cover the network of abs with GPCR abs as prominent players.

Keywords: G protein-coupled receptors, anti-G protein-coupled receptor antibodies, immune cell homeostasis, precision medicine, angiotensin receptor 1

G PROTEIN-COUPLED RECEPTOR SIGNATURE REFLECTS THE INDIVIDUAL RESPONSE TO CHANGING ENVIRONMENTAL CONDITIONS

Life requires recognition, response, and adaptation to changing conditions. Our ability to react to environmental stimuli, such as to taste and smell, indicates the need for a very sensitive system. G protein-coupled receptors (GPCRs) are crucial for these processes. They also regulate mood and behavior as well as our immune system (1–3). GPCRs are placed on cell membranes and are thus exposed to the variable and changing extracellular milieu. If not activated, GPCRs are thought to

exist in a conformational equilibrium between active and inactive biophysical states (4). The flexible response to fluctuating environmental factors, a hallmark of GPCRs, is based on their capability to bind variable ligands, to change their conformations, to up- or down-regulate their membrane expression, and to complex with other proteins and receptors forming homo- or heterodimers. As consequence, GPCR functionally interact with a broad range of extracellular and intracellular proteins including signaling molecules as well as other membrane proteins. An example for close functional and local protein interactions starting with GPCR-mediated signaling is the activation of the GPCR dopamine 4 receptor stimulating platelet-derived growth factor receptor β (PDGFRβ) to inhibit the N-methyl-D-aspartate receptor (NMDAR) activity (5). However, even under physiological conditions, the environment is fluctuating in a certain range. Therefore, at each time point, a unique GPCR expression can be assumed to reflect the unique current micro and macro environment. Accordingly, GPCR expression and in sum the GPCR signature could image the cellular exposome. Functionally, changes in GPCR expression have consequences: They determine the cellular function *via* signaling as well as by affecting the binding capacity of their ligands.

Around 800 GPCRs have been described so far. Prominent examples of GPCRs are adrenergic receptors, cholinergic receptors, or proteinase activated receptors. GPCRs are widely expressed on immune cells.

G PROTEIN-COUPLED RECEPTOR ANTIBOIDES OFTEN CORRELATE WITH EACH OTHER'S AND THUS CONNECT DIFFERENT PROTEINS

Antibodies (abs) such as GPCR abs are a feature of the adaptive immune system and have been described first in vertebrates (2, 6). Based on the complex nature of GPCRs with three intra- and extracellular loops and intra-membranous domains, it is a challenge to develop test assays against the native structure. However, by using membrane extracts from cells overexpressing a specific GPCR, the levels of the abs can be sensitively measured by ELISAs. As recently shown by studying more than 30 abs to dopaminergic, serotoninergic, muscarinic, adrenergic, vascular, and immune receptors in humans, distinct correlations of the antibody levels have been identified. In addition, some GPCR abs also correlate with abs against growths factors and their receptors. The presence of a physiological antibody network of GPCR abs as central players, but also of other abs, is probably best reflected by using the term antibodiom. However, the correlations of the abs are slightly different between male and female healthy donors or between young and old persons. In general, old males revealed the highest number and strengths of correlations between various abs (2, 6). As example, abs against the angiotensin receptor type-1 (AT1R) strongly correlate with abs against the endothelin receptor type-A (ETAR). Abs against the complement C3a receptor 1 (C3AR1) correlate with F2R coagulation factor II receptor (PAR1) abs, and CXCR3 abs strongly correlate with CXCR4 abs (2). As shown before, both C3AR1 and F2R expressions are upregulated upon stimulation (7). In addition, CXCR3 as well as CXCR4 were shown to be strongly co-localized in the presence of inflammation (8). Therefore, and although the structural basis for these ab correlations is not known so far, it is very likely that they represent the expression status of their corresponding receptors and thus, indirectly, the exposome (9–11). In line with this, increased expressions of AT1R in peripheral blood mononuclear cells (PBMC), in the skin, as well as in the lung corresponded to increased AT1R ab levels in patients with systemic sclerosis (SSc), a severe autoimmune disease (12–17).

G PROTEIN-COUPLED RECEPTOR ANTIBOIDES OFTEN CORRELATE WITH EACH OTHERS AND THUS CONNECT DIFFERENT PROTEINS AUTOANTIBODIES AND THE ANTIBODIOM AS A LONG-TERM IMAGE OF THE INTERPLAY BETWEEN THE CELLULAR EXPOSOME AND THE INDIVIDUAL IMMUNE RESPONSE

As stated above, GPCRs can respond very fast to environmental factors. In contrast, changes in the adaptive immune system take much more time: GPCR abs are of the IgG subtype, which requires T cell help and class switches (18, 19). Our studies indicate that GPCR abs changed in a time frame of months or years (2). In other words, the GPCR abs are less sensitive as the GPCR to respond to acute changes in the environment. Therefore, GPCR abs represent most likely the chronic GPCR expression and activation status (GPCR signature). Accordingly, chronic cellular environmental factors (the chronic individual exposome) are reflected by the GPCR as well as the GPCR ab signature. This translation of the environment into the immunological memory *via* the antibodiom could be a novel mechanism for the interaction between the environment and the immune system. So far, studies on the interplay between GPCR abs and environmental conditions have just started.

Immune responses to antigens are variable between individuals based on different HLA haplotypes, which determines the antigen presentation to T cells. In addition, several genes involved in the very tight regulation of the immune response show polymorphisms. Therefore, the antibody generation including the immune response to GPCRs mirrors the individual immune system. In addition, the antibody network or the antibodiom reflects important individual processes, which makes it interesting for precision medicine.

INCREASED AND DECREASED G PROTEIN-COUPLED RECEPTOR ANTIBODY LEVELS AS MARKERS FOR AUTOIMMUNE AND NON-AUTOIMMUNE DISEASES

Increased abs against self-antigens are a feature of autoimmune diseases. Indeed, high GPCR abs are associated with several rheumatic autoimmune diseases such as with SSc, systemic lupus

erythematosus (SLE), or primary Sjogren's syndrome (2, 18–21). In SSc, AT1R abs as well as ETAR abs are increased and predict vascular complications such as pulmonary arterial hypertension as well as mortality and response to therapies (18, 19). Nevertheless, higher GPCR abs compared to healthy individuals are common in human pathophysiology and were also found in endocrinological diseases (Graves' Disease or Hashimoto thyroiditis), in gynaecology (preeclampsia), cardiac diseases (heart transplantation, cardiomyopathy, chronic heart failure, orthostatic hypotension, and postural tachycardia syndrome), or in neurologic diseases such as in dementia, Alzheimer Disease, chronic fatigue syndrome (myalgic encephalomyelitis), or in complex regional pain syndromes (6, 13, 20–23).

In contrast, some diseases including autoimmune diseases are characterized by reduced levels of GPCR abs when compared to healthy donors. Thus, patients with giant cell arteritis have lower ETAR ab levels compared to healthy donors (24) and patients with granulomatosis with polyangiitis have reduced levels of antibodies against complement receptors (2). Furthermore, reduced levels of antibodies against ß1-adrenergic receptors are present in patients with acute coronary syndrome and are associated with a more severe disease particularly with a higher risk for early reinfarction and cardiovascular death in patients ≤ 60 years (25, 26). Lower abs against the thrombin receptor PAR-1 are associated with ovarian cancer and with high-grade carcinoma (2, 27). Finally, decreased levels of abs against the chemokine receptor CXCR3 and CXCR4 are found in patients with progressive interstitial lung disease (28, 29).

The presence of both increased as well as reduced GPCR ab levels further strengthen the idea of the presence of physiological levels and a balanced generation of autoantibodies in human physiology and pathophysiology.

CORRELATIONS OF ANTIBODIES AS MARKERS FOR AUTOIMMUNE AND NON-AUTOIMMUNE DISEASES

In addition, by studying the levels of multiple abs by ELISA tests in different diseases, characteristic alterations in the antibody correlations were identified, which differed in comparison to healthy donors (2, 13). In SSc, correlations changed between different GPCR abs, but also between GPCR abs and those directed to growth factors and their corresponding receptors (Tyrosine kinase receptors etc). In patients with SSc, new antibody correlations appeared between abs targeting AT1R, epidermal growth factor receptor (EGFR), and vascular endothelial growth receptors (VEGF-R1 and VEGF-R2) in comparison to heathy donors. Specific correlation changes were also observed in patients with Alzheimer Disease between neuronal receptors and growth factor receptors. Here, the dopaminergic and serotonergic ab pattern was associated with increased mortality, the cholinergic receptor pattern correlated with increased mood symptoms, and both of them were different to healthy controls (2, 3). Ovarian cancer was characterized by strong antibody correlations between different growth factors (2). Correlation changes could reflect changes in the GPCR expression pattern with hetero- and homodimerization and receptor co-expressions as well as interactions of GPCRs with other proteins, receptors, or signaling molecules. From an immunological point of view, changes in the antibody correlations could reflect epitope spreading and the emergence of novel so far hidden epitopes induced by acquired, exposome-induced changes in the GPCR signature. This break of tolerance leads to the generation of novel GPCR abs.

So far, it is not known whether these antibody correlations have an impact on the development of the different diseases. Only few functional studies exist analysing cross activation of the corresponding receptors by the correlating abs. Here, AT1R abs were shown to activate the ETAR and vice versa (30, 31) indicting functional consequences of ab correlations. However, ab levels as well as the ab correlations (the individual antibodiom) can serve as biomarkers for diseases.

G PROTEIN-COUPLED RECEPTOR ANTIBODIES ARE SPECIFIC G PROTEIN-COUPLED RECEPTOR LIGANDS CAUSING UNIQUE CELLULAR EFFECTS AND DISEASES

In the last decades, several GPCR abs were linked to the pathogenesis of very heterogenous diseases present in different medical disciplines. Sterin-Borda et al. described the effects of beta adrenergic abs as well as of other antibodies as potential drivers for Chagas disease (32). Wallukat et al. discovered functional beta-1 adrenergic receptor abs in patients with idiopathic dilated cardiomyopathy (33). AT1R abs are risk factors for rejection after solid organ transplantations and were shown to be drivers for non-HLA-dependent transplant rejections (21, 23, 34–36).

GPCR abs specifically bind to their corresponding receptors, which could have functional consequences as identified for several GPCR abs. As studied particularly in Graves' Disease, neutral, stimulating, and blocking thyrotropin receptor (TSHR) abs have been described, which may compete for receptor binding with overlapping epitopes (21, 37–41). Here, stimulating TSHR abs cause hyperthyroidism and induction of a unique syndrome, which is different from sole hyperthyroidism initiated by the presence of increased peripheral hormones (41). *In vitro*, abs from SSc patients stimulated various cytokines (e.g. TGFß, IL-6, CCL18), chemokines (e.g. MCP1, IL-8), and adhesion molecules (VCAM-1) in cells such as in immune cells or in resident cells e.g. in microvascular dermal endothelial cells or in fibroblasts by signaling (18, 19, 22, 42) The reduction of the effects by AT1R and/or ETAR blockers indicates a contribution of anti-AT1R/ETAR abs. Indeed, our recent studies in mice proved the causal role of AT1R abs for interstitial lung disease, skin inflammation and skin fibrosis and, under certain circumstances, for obliterative vasculopathy (unpublished).

Thyrotropin receptor abs as well as AT1R abs are examples for stimulating and agonistic antibodies (6, 18, 41, 42). For most GPCR abs, the effects on their corresponding receptors still need to be deciphered.

Concerning the functional role of the antibodiom, specific effects of abs were also obtained by passive transfer of IgG fractions from SSc patients. Here, interstitial lung disease and vasculopathy, clinical signs of SSc, were induced (31). Although studied, no cytotoxic effects of the abs or complement activation have been observed. In addition, we recently have analysed the secretion of proteins upon stimulation of monocytes with purified IgG fractions from SSc patients. The secretome induced by IgG from patients was different to this from healthy donors and revealed associations with clinical symptoms (43). Other disease control studies are ongoing.

G PROTEIN-COUPLED RECEPTOR ANTIBODIES CONTROL IMMUNE CELL HOMEOSTASIS AND AVOID SYSTEMIC IMMUNE RESPONSE TO ACUTE LOCAL DAMAGE

GPCRs are often expressed on immune cells whereas the expression of GPCR in tissues is very differently distributed. Some GPCRs are expressed mainly in a single tissue such as the thyrotropin receptor. Others such as AT1R are expressed in several organs (12, 14–16). The presence of a natural network of functional and regulatory abs could be important for several reasons: They stimulate immune cells to express cytokines and chemokines (17, 42). Therefore, GPCR abs may contribute to physiological blood levels of these mediators. In addition to the direct ab effects to the corresponding receptor *via* signaling, GPCR abs also attract cells expressing the corresponding receptors like other ligands. This was shown for abs against the endothelin receptor type-A (ETAR), AT1R, or the chemokine receptors CXCR3 and CXCR4 (2, 16, 17, 29). Therefore, both the abs as well as the GPCR ligands likely attract immune cells and, vice versa, the abs are attracted by the immune cells. This assumed equilibrium or steady state could be important for immune cell homeostasis between blood and tissues (**Figure 1A**). Accordingly, serum concentrations of ligands and abs could determine the threshold for tissue invasion to avoid an inadequate immune response to local tissue damage.

ABNORMAL HIGH G PROTEIN-COUPLED RECEPTOR ANTIBODIES AND G PROTEIN-COUPLED RECEPTOR TISSUE EXPRESSION CAUSE CELL INVASION

According to the concept of a balance between GPCR abs and GPCR tissue expression, both the presence of abnormally high specific GPCR abs and abnormally high tissue expression of the corresponding receptor may cause immune cell invasion, which can be harmful (**Figure 1B**). Examples for the pathogenic effects of high GPCR abs are discussed before such as for Graves' disease showing increased TSHR abs also leading to tissue inflammation (41). In addition, GPCR expression in the tissues can become upregulated e.g. by acute infections or in chronic conditions: Thus, increased lung AT1R expression was shown to be present in acute respiratory distress syndrome or acute lung injury induced by E. coli (44). Depending on the local expression level of AT1R (which probably corresponds to the degree of harm), AT1R abs as ligands will accumulate in the tissue with increased expression of AT1R, bind and activate AT1R (e.g. on fibroblasts) and will thus initiate signaling for the induction of chemokines. This could contribute to an adequate and balanced immune cell invasion, which is, under physiological conditions, necessary to couple with causes of harm.

In addition to microbes, exposure to diesel increased ETAR expression in the lungs of mice (45). In SSc, associations are present between the severity of interstitial lung disease (ILD) with the exposure to Benzol, which is another surrogate marker for traffic-induced air pollution (46). Here, endothelin receptor expressions are increased in the lungs (47).

Based on the nature of GPCR to respond to changing conditions, barrier organs such as lungs, the GI tract, or the skin might be more vulnerable to chronic environmental exposures (44, 45, 47). In addition, in barrier organs, the distance between blood and environment is low. Invasion of immune cells will certainly affect the normal function e.g. of the alveolar membrane.

POSSIBLE CONSEQUENCES OF LOCAL IMMUNE CELL INVASION

Moreover, the accumulation of specific GPCR+ immune cells in the tissues and therefore, of the corresponding antigen, might also stimulate an immune response e.g. to AT1R leading to T cell help and the generation of AT1R+ B cells and plasma cells, which will locally produce AT1R abs. In our mouse model, we have partially seen germinal centre-like structures as well as ab binding (unpublished). So far, the specificity of the local tissue abs needs to be tested and this is an ongoing project in our studies. However, severe and uncontrolled tissue inflammation will result in a high consumption of cytokines such as of interleukin-2 (IL-2), which can lead to a local deficiency of regulatory T cells strongly requiring IL-2 (48). This could drive epitope spreading and autoimmunity to other autoantigens such as to down-stream signaling molecules of the activation cascade or to nuclear proteins as shown before in lupus (49). Theoretically, this could explain the generation of typical marker antibodies such as centromere abs in SSc. However, the hypothesis that GPCR abs initiate the immune response to the classical autoantigens needs to be proven by further studies.

ABNORMALLY REDUCED G PROTEIN-COUPLED RECEPTOR ANTIBODIES AS BIOMARKERS FOR ACUTE DAMAGE AND POSSIBLE DRIVERS FOR VASCULAR DAMAGE

In comparison to healthy donors, GPCR ab levels can also be reduced. As mentioned above, GPCR ab levels are in a tight balance with the general and chronic expression status of their corresponding GPCR, which can be up- or downregulated. Downregulation could result in a reduced GPCR ab response. In addition, some GPCRs are nearly

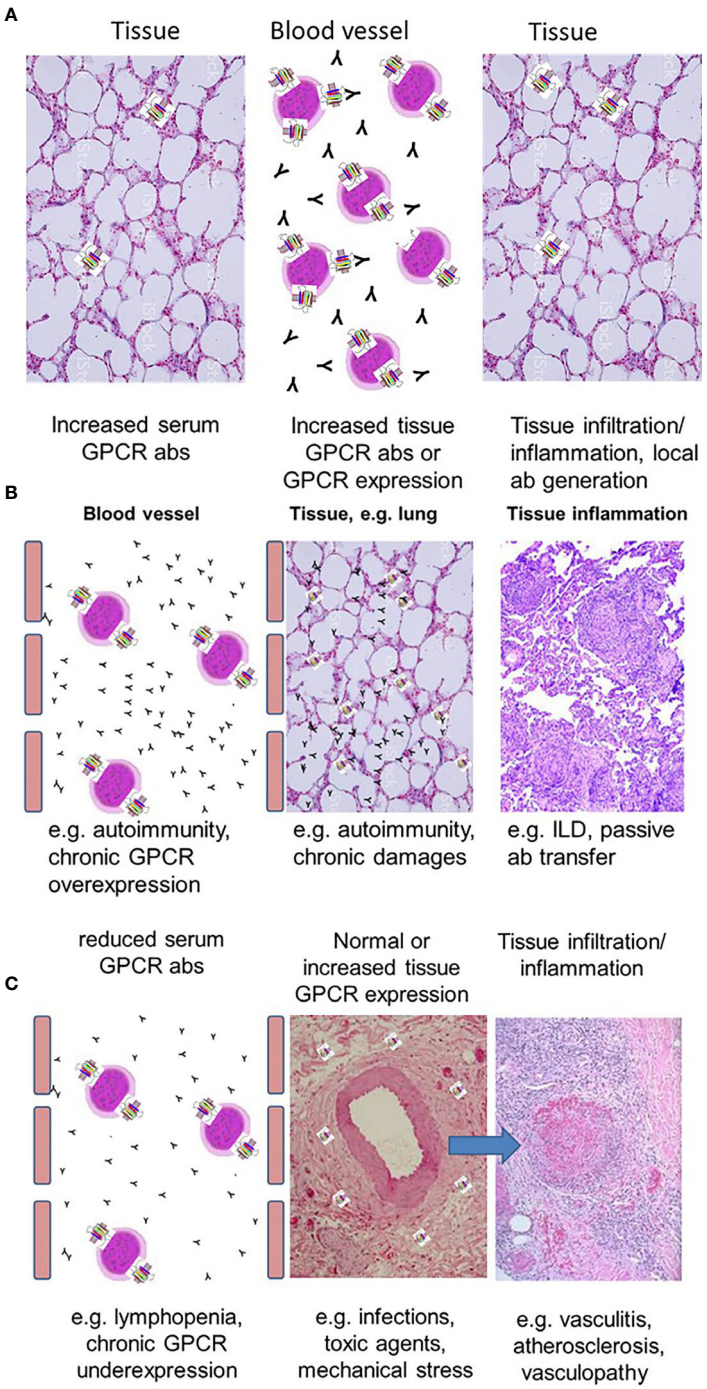

FIGURE 1 | Concepts of altered GPCR abs in tissue inflammation. **(A)** Schematic presentation of a normal immune cell homeostasis with a tight balance between the levels of GPCR abs and GPCR expression of immune cells and tissue-resident cells. **(B)** Concept to explain tissue inflammation by either increased GPCR abs or increased tissue expression of GPCR. **(C)** Reduced GPCR ab levels could also cause tissue damage based on deficient competition to local tissue chemokines.

exclusively expressed on immune cells. Examples for GPCRs mainly expressed by immune cells are the complement receptors 3 and 5 expressed on monocytes and macrophages. Another example is the chemokine receptor CXCR3, which is highly expressed on recently activated T cells. Under physiological conditions, the levels of the antibodies are low. Upon T cell activation, both CXCR3 ab levels as well as the serum concentrations of chemokines define the threshold for the migration of CXCR3+ T cells into the tissues by competing for receptor binding. Depending on the severity of damage, the number of tissue immune cells expressing CXCR3 could overreach the

number of immune cells expressing CXCR3 in the blood. In those conditions, GPCR abs could become attracted to go into the tissues to bind their specific receptor or *via* cross-reactivity, other proteins. This concept could explain abnormally reduced ab levels in the blood as also shown for e.g. ß1 adrenergic receptor and ETAR abs in acute coronary syndrome or acute vasculitis flares, respectively (19, 20). Therefore, reduced ab levels could be a marker for a more acute tissue damage or of an acute flare (**Figure 1C**) and might contribute to vascular inflammations. This discussed scenario is still a hypothesis based on several associations, which needs to be proven. In addition, the identification of reduced GPCR ab levels by test assays is a challenge as well as the identification of the ab function for signaling. **Figure 2** provides a summary of the GPCR-GPCR ab interactions and the pathophysiological consequences.

G PROTEIN-COUPLED RECEPTOR ANTIBODIES AS TARGETS FOR FUTURE THERAPIES

Unfortunately, GPCR abs are very resistant to immunosuppression as shown for AT1R abs in SSc patients (19) probably based on the ongoing presence of the exposome or the driving environmental factors, of the corresponding GPCR signature, and of the individual immune system. Therefore, reduction of abnormal high GPCR ab levels requires aggressive therapies such as autologous stem cell transplantation or combination of immunosuppressants (50–52). Both strategies are used in severe autoimmune diseases. To target the ab-mediated GPCR activation or ab-receptor-interaction more specifically, ab-neutralizing peptides or monoclonal idiotypic antibodies could be successful, which still needs to be shown. Although successful in animal models, small molecule inhibitors for the TSRH were not transferred into the clinic based on cost-effective alternatives (53). However, this could be different in other diseases such as in SSc or transplant rejection. Very recently, aptamers have been introduced as promising tools in nanomedicine. These small single-stranded DNA or RNA molecules can be used for the effective binding and removal of proteins such as antibodies. Aptamers specifically binding to some GPCR abs are currently investigated in animal studies showing promising data (54).

The presence of a functional and physiological network of abs and of a specific antibodiom is a theoretical basis for the use of intravenous or subcutaneous immunoglobulins in severe inflammatory diseases. The antibodies could reconstitute immune

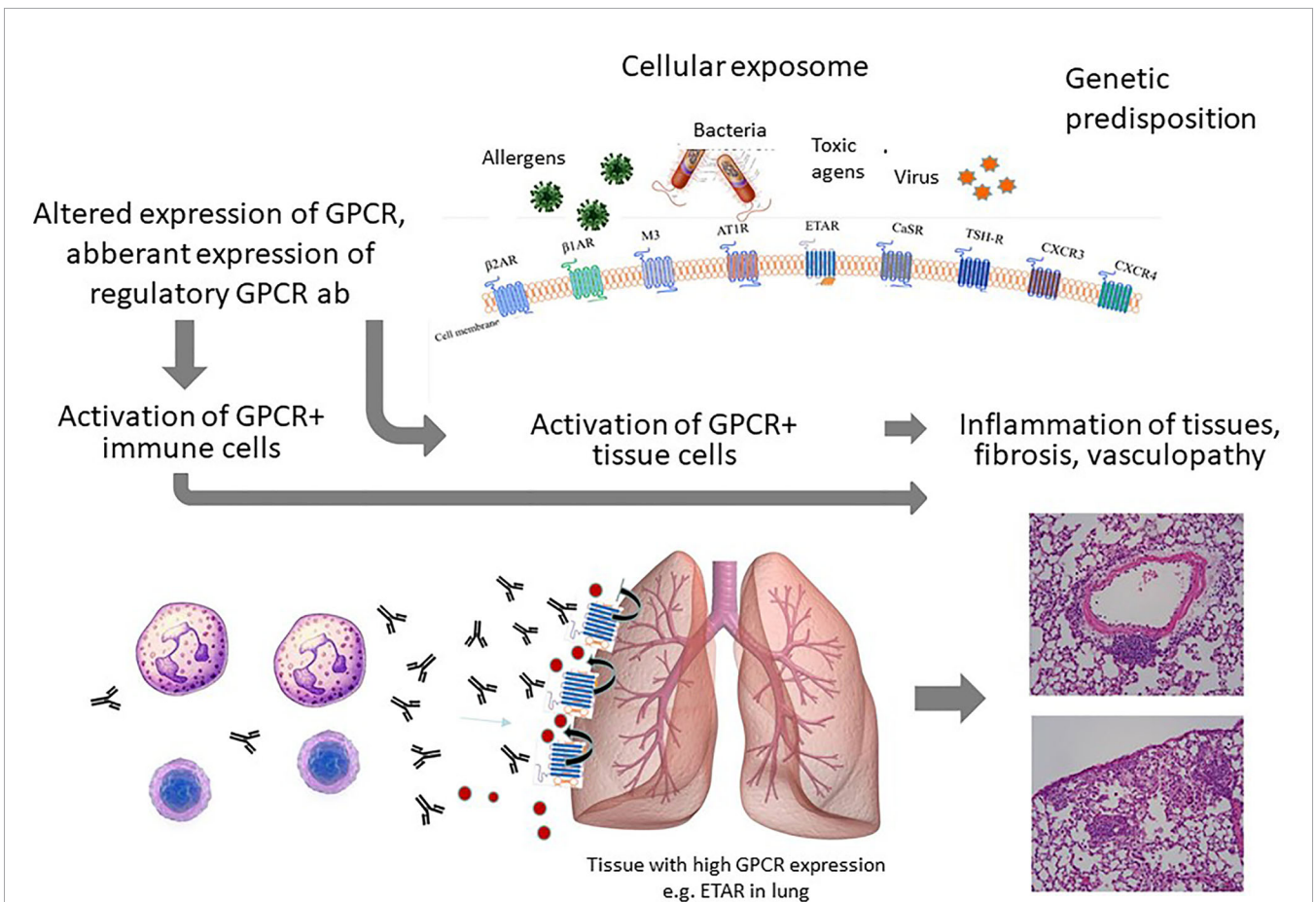

FIGURE 2 | Summary of the concept: The chronic cellular exposome leads to an altered GPCR and GPCR ab signature. The ab effects on activation/migration of immune cells as well as tissue resident cells will lead to inflammation and vasculopathy as illustrated here for the lung.

cell homeostasis. However; for long-term efficacy, very cost-intensive long-term and repetitive IVIG doses are required as shown for other diseases with established long-term IVIG therapies (49, 55). In addition, the ab-induced signaling could identify novel therapeutic targets as shown in a very recent manuscript identifying the AP1-pathway for the expression of the profibrotic cytokine CCL18 induced by abs (43). So far, current therapies target the binding and signaling of the natural ligands. The important role of GPCR abs in the pathogenesis of diseases and, therefore, as effector molecules, implicate to target the interaction of GPCR abs with their corresponding GPCR for future therapies.

FURTHER POTENTIAL RESEARCH FIELDS

GPCRs are also expressed on cancer cells. Therefore, GPCR abs could also be involved in the homeostasis of these cells and in the generation of metastases (56). In addition, they also may influence the reaction of the immune system to viral or bacterial agents, which emerge as an interesting field in the current pandemic.

CONCLUSION

GPCR abs are special since they could reflect the interplay of the individual immune system with the individual exposome including the internal milieu. GPCR abs are together with the antibodiom biomarkers for autoimmune and non-autoimmune diseases. Based on their functions as ligands and regulators of the cell homeostasis, they can be used to identify further biomarkers, pathways, and therapeutic options. Specific GPCR abs cause and modulate diseases as shown so far for several GPCR abs. The identification of specific GPCR ab functions as well as the interplay between various antibodies could help to decipher diseases and their mechanisms. At best and hopefully, this provides a better approach to understand diseases. The future goal for the pharmaceutical industry should be to interfere with the interaction between abs, the physiological ligand, and their corresponding GPCR to develop novel drug candidates.

AUTHOR CONTRIBUTIONS

GR wrote the first draft, reviewed the manuscript, researched for this, wrote the last version, correspondence, ideas, and concept. FP reviewed the manuscript and graphic design. HH contributed ideas and concept, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Stem Cells and Organoid Technology in Precision Medicine in Inflammation: Are We There Yet?

Florian Tran[1,2], Christine Klein[3], Alexander Arlt[2,4], Simon Imm[1], Evelyn Knappe[3], Alison Simmons[5,6], Philip Rosenstiel[1] and Philip Seibler[3]*

[1] Institute of Clinical Molecular Biology, University of Kiel, Kiel, Germany, [2] Klinik für Innere Medizin I, Universitätsklinikum Schleswig-Holstein, Kiel, Germany, [3] Institute of Neurogenetics, University of Lübeck, Lübeck, Germany, [4] University Department for Gastroenterology, Klinikum Oldenburg AöR, European Medical School (EMS), Oldenburg, Germany, [5] MRC Human Immunology Unit (MRC), University of Oxford, Oxford, United Kingdom, [6] Translational Gastroenterology Unit, University of Oxford, Oxford, United Kingdom

Individualised cellular models of disease are a key tool for precision medicine to recapitulate chronic inflammatory processes. Organoid models can be derived from induced pluripotent stem cells (iPSCs) or from primary stem cells *ex vivo*. These models have been emerging over the past decade and have been used to reconstruct the respective organ-specific physiology and pathology, at an unsurpassed depth. In cancer research, patient-derived cancer organoids opened new perspectives in predicting therapy response and provided novel insights into tumour biology. In precision medicine of chronic inflammatory disorders, stem-cell based organoid models are currently being evaluated in pre-clinical pharmacodynamic studies (clinical studies in a dish) and are employed in clinical studies, e.g., by re-transplanting autologous epithelial organoids to re-establish intestinal barrier integrity. A particularly exciting feature of iPSC systems is their ability to provide insights into organ systems and inflammatory disease processes, which cannot be monitored with clinical biopsies, such as immune reactions in neurodegenerative disorders. Refinement of differentiation protocols, and next-generation co-culturing methods, aimed at generating self-organised, complex tissues *in vitro*, will be the next logical steps. In this mini-review, we critically discuss the current state-of-the-art stem cell and organoid technologies, as well as their future impact, potential and promises in combating immune-mediated chronic diseases.

Keywords: stem cell, cancer, precision medicine, co-culture, host-microbe, immune-epithelial interactions, induced pluripotent stem cells, patient derived organoids

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****Correspondence:***
Florian Tran
f.tran@ikmb.uni-kiel.de

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STEM CELLS AND ORGANOIDS – THE RISE OF NOVEL MODEL SYSTEMS

Chronic inflammatory diseases are characterised by either a persisting stimulus for inflammatory signals and/or an inadequate resolution of a response to tissue insults, leading to the development of cancer, neurodegenerative or autoinflammatory disorders. In this complex, disharmonised multi-cellular immune response, the epithelium, immune cells and the microbiome play central roles and thus the development of adequate *in vitro* models of disease, reflecting the complexity of immune interactions in chronic inflammation, is an urgent need in all biomedical fields. However, tissue biology is very

challenging to study in mammals, and progress can be hindered by sample accessibility and ethical concerns in humans. One of the cardinal concepts underlying organoid technology is the idea that stem cells have the intrinsic ability to self-organise into 3D structures that resemble *in vivo* organs. A major breakthrough was achieved in 2009, when adult tissue-resident stem cells were found to proliferate and self-organise, *in vitro*, into organoids (1, 2) (**Figure 1A**). The method has since been adapted to generate murine and human organoids from epithelial tissues of major organs like skin, kidney, liver and intestine (3–6) (**Figure 1B**), and used for various physiological and disease-related studies, e.g. for complex disorders like inflammatory bowel disease (IBD). Using intestinal organoids, the functional link between the IBD risk gene *ATG16L1*, interleukin(IL)-22 signalling and STING-dependent type-I-interferon (IFN-I) response was discovered (7). Similarly, the role of the DNA damage repair gene *RNASEH2B* in intestinal inflammation and tissue regeneration was identified in organoid models (8). Purified intestinal epithelium from inflamed intestinal tissue obtained from IBD patients displayed distinct epigenetic and transcriptional alternations, which were retained in organoid cultures and correlated with disease outcome (9). Inflamed IBD tissue-derived organoids display increased activation of typical molecular hallmarks of IBD such as the ATF6 pathway as a branch of the unfolded protein response (UPR), which were used to identify novel anti-inflammatory ATF6 targeting compounds (10). As organoids can provide experimental manipulability and maintain at the same time biologic complexity, they function as a bridge between conventional 2D cell culture and animal models (2).

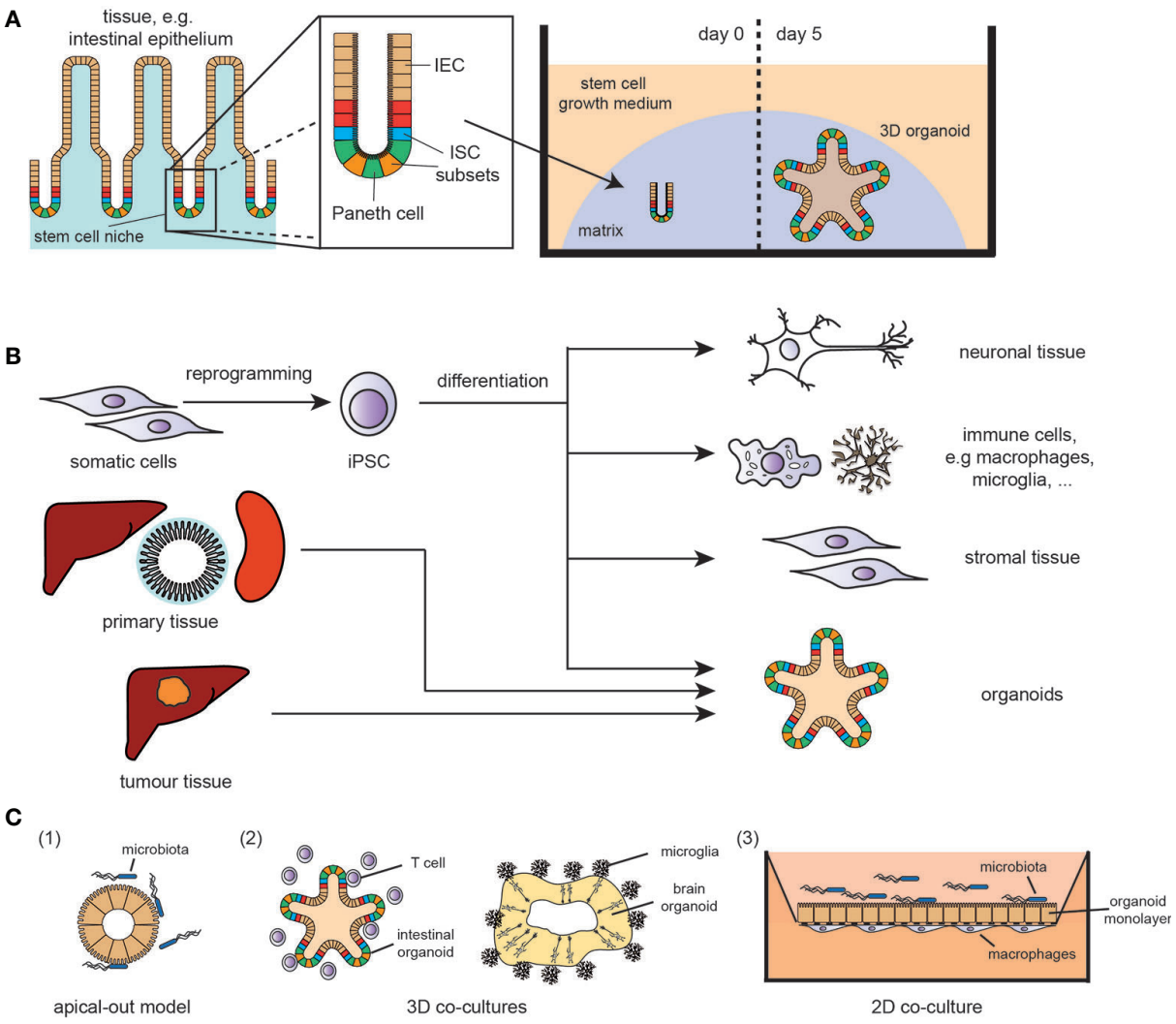

FIGURE 1 | **(A)** Example of adult tissue-derived organoids (here: small intestine); IEC, intestinal epithelial cells; ISC, intestinal stem cell. **(B)** Overview of stem cell- and (tumour) tissue-derived *in vitro* models (selection); iPSC, induced pluripotent stem cells. **(C)** Overview of co-culture systems (simplified selection): (1) apical-out model of intestinal organoids for host-microbe-interaction studies, (2) 3D co-culture systems of immune cells with organoids, e.g., T cells/intestinal organoids and microglia/brain organoids, (3) 2D co-culture systems with a polarized organoid monolayer with either immune cell (basal interface) or microbe (apical interface).

A further breakthrough was the development of human induced pluripotent stem cell (iPSC)-derived organoid culture. Intestinal organoids were one of the first iPSC-derived tissues consisting of a polarised, columnar epithelium patterned into villus-like structures and crypt-like proliferative zones (11). The iPSC technology has the enormous potential to overcome the limitations of accessibility of specific tissues, such as brain or heart, providing improved patient-derived cellular models of human disease that can also be used for drug screens and personalised treatment strategies (**Figure 1B**). Cerebral brain organoids displayed various discrete though interdependent brain regions (12). This potential is further strengthened by combining iPSC technology with genome engineering - allowing the correction of mutations in patient-derived iPSCs (13) and the modification of reporter lines, thereby facilitating differentiation towards specific cell types (14).

In the following, we will discuss the technical progress and remaining limitations on using more complex cell culture models to understand inflammatory disorders, host–pathogen interactions or cancer.

PRECISE DISEASE MODELLING: CO-CULTURES AND TISSUE ENGINEERING TO STUDY AUTOIMMUNE DISEASES AND INFECTIONS

A wide range of co-culture and organoid-based disease models that reproduce genetic immune diseases (7, 8, 15) and host–pathogen interactions (16, 17) have already been developed and provide proof of principle that these complex cultures can show certain well-known pathological features. These model systems are the result of tremendous advances and bioengineering innovations of the past decade.

Impaired epithelial host-microbe interaction is one of the important features of IBD and thus, model systems focusing on this interplay are needed (18). Due to the "apical-in/basolateral-out" polarity of conventional organoid systems, microbes need to be microinjected into the lumen of the organoids to mimic physiological host-microbial cross-talks (19–21). This technique however is slow, even with standardised high-throughput approaches technically very challenging and the reproducibility of the results is low due to the heterogeneity of the organoids and their luminal content (22).

To combat this issue of "wrong" epithelial polarity, an "apical-out" system with reverse organoid polarity was developed (22) (**Figure 1C**), providing a more suitable and reliable model for processes and properties of the epithelium, e.g. examining nutrient absorption and host-pathogen interactions, such as inflammatory cytokines/chemokines, antimicrobial peptides and ROS production.

Another approach to assess host-pathogen interactions is the co-culturing of an epithelial monolayer of preserved apical-basolateral polarity with selected pathogens (**Figure 1C**). Using such models, the role of *K. pneumoniae* in priming T_H17 response in primary sclerosing cholangitis (15) or the pathophysiology of chronic *Helicobacter pylori* infections (20, 23) were identified. Recently, microfluidic-based systems, i.e. the *human microbial crosstalk* (HuMiX) system (24, 25), were established, enabling co-culturing of human intestinal epithelial monolayers with anaerobic pathogens and the analysis of diet-microbiome-human interactions. Besides studies on antimicrobial response, the development of two-dimensional organoid cultures with an air-liquid-interface increased the complexity and maturation of the epithelial layer, providing a better approximation to the *in vivo* tissue, similar to colonic monolayers (26), and led to the discovery of a novel injury-related cell type, associated with IBD-related tissue regeneration.

The interaction of the epithelium with the stroma, including immune cells, mesenchymal cells and neuronal cells, is vital for organ development and homeostasis and needs to be taken into account when modelling complex immunological diseases like IBD (27). Stromal cells provide the niche for stem cell growth by production of ECM and secretion of essential growth factors, such as WNT proteins (27) and R-spondin (28). Various *in vitro* models (both 2D and 3D) have been co-cultured with different types of immune cells to assess immune-related orchestration of the epithelial barrier and vice versa the communication back to the specialised immune cells (29). E.g., co-culturing of intestinal organoids and macrophages led to enhanced barrier integrity (30), and the epithelial-macrophage communication enabled a more coordinated immune response to infection.

The enteric nervous system (ENS), controlling several functions, such as motility and permeability, has been linked to enteric neuropathies and gut disorders (31). To that end, a model of co-culturing of intestinal epithelial cells with ENS neurons and glia or subepithelial myofibroblasts was developed (32), showing that the ENS influences intestinal stem cell (ISC) fate by increasing differentiation towards the enteroendocrine lineage and thereby modulates intestinal barrier function. Vice versa, immune cells like tissue resident macrophages upon gastrointestinal infections modulate cellular fate of enteric neurons (33). Going one step further, human iPSC-derived intestinal tissue with a functional ENS was generated by combining intestinal organoids with neural crest cells, in order to investigate the pathophysiology of *Hirschsprung's disease* (31). This model could also provide a platform for analysing the epithelium/ENS-axis in IBD.

Vice versa, environmental factors influencing the microbiome (e.g. intestinal infections) are suggested to play a key role in the initiation and progression of neurological disorders, such as Parkinson's disease (PD) (34–36). PD is characterised by the loss of dopaminergic neurons and intracellular inclusions composed mainly of alpha synuclein, which can be identified in intestinal biopsies years prior to PD diagnosis (37). Notably, it was shown that inflammatory processes, e.g. mitochondrial stress-associated STING/IFN-I response in the absence of PD-linked Parkin or Pink1 function, are drivers of PD pathology (36, 38). Therefore, it is of interest not only to examine disease mechanisms of the gut-brain axis but also direct inflammatory processes in the brain triggered by microglia activation (39). In this regard, brain organoids in co-culture with microglia represent a major breakthrough in neuronal disease modelling techniques (40) and will serve to facilitate the development of more precise human brain models for basic mechanistic studies in neural-glial interactions and drug discovery.

There remains one crucial factor none of these models accounts for, that is blood vessels. Aside from transporting the vital contents of our blood to and from the demanding organs, vascularization is a critical component of physiological (and pathological) development. Recently, human pluripotent stem cells were engineered to induce endothelium development by expressing ETS variant 2, which contributed to forming a complex vascular-like network in human cortical organoids (41). Vascularized organoids displayed enhanced functional maturation and acquired several blood-brain barrier characteristics, including an increase in the expression of tight junctions, nutrient transporters and trans-endothelial electrical resistance. These cultures formed vasculature-like structures that resemble the status in early prenatal brain and present a robust model to study disease *in vitro*.

The key challenge of the next years will be the establishment of multi-compartment *in vitro* models for disease and beyond the two-compartment organoid co-culturing systems [e.g. organ/body-on-a-chip, including tissue cultures from multiple organs (42, 43)], to really represent complex, multi-organ immunological disorders.

PATIENT IN A DISH: EXCURSUS ON PREDICTING THERAPY RESPONSE IN INFLAMMATION-DRIVEN CANCER

Inflammation is a driving aspect of oncogenesis and a necessary component of the established tumour's mechanism of resistance. This aspect of the immune-oncological crosstalk has recently gained attention due to the use of immunotherapy in nearly all type of cancer (44). Previous oversimplified cell culturing methods lack the tumour's micro-environment, which have only been partially resolved by co-culturing models of tumour cell lines with other somatic cells (45–47) (**Figure 2A**). Animal models, including genetic tumour models (GEM), murine-derived organoids (MDO) and xenotransplants, display higher degrees of tumour complexity, but are limited by inter-species differences in immunological mechanisms and chemokine signalling, compared to human cancer (48).

The possibility to easily generate primary tissue-derived organoids provides the basis for personalised *ex vivo* models, particularly in cancer research. Cancer-derived organoids display several traits that are similar to the original tumour and therefore allow the understanding of cancer biology by large-scale tumour bio-banking and high-throughput drug screenings, which gave rise to the discovery of novel anti-tumour compounds (49).

The availability of CRISPR/Cas9 systems enabled genetic engineering on (cancer) organoids with, however, highly variable efficiency of genome editing, depending on the route of CRISPR-Cas9 installation (50). As a proof of concept, sequential introduction of hallmark colorectal cancer mutations into human colonic organoids licenced growth factor independent proliferation of the organoids and mimicked aspects of the classical adenoma-carcinoma sequence (51). In the same study, re-implantation of these transformed organoids in a murine metastasis model demonstrated that, beside canonical cancer mutations, additional molecular lesions as chromosomal instability are necessary for an invasive cancer behaviour. Another example from the same group is the generation and usage of CRISPR/Cas9-guided knock-in of LGR5- and KRT20-reporters into cancer organoids to trace tumour stem cell behaviour *in vivo* (52).

On an individualised level, patient cancer-derived organoids (PDO) are suitable in the prediction of therapy response of an individual tumour or metastases by high-throughput screenings of therapies (e.g. compound screens, chemotherapy, irradiation) on a small specimen in neo-adjuvant settings (53, 54).

To depict the complexity of tumour microenvironments, including immuno-oncological cross-talks, distribution of drugs and metabolites in the tumour etc., the need for cancer organoid models exceeding the pure epithelial tissue was recently defined (55). The following two strategies are pursued and include the tumour epithelium and tumour-associated cell types such as, immune cells or stroma:

i. The cultivation of organotypic tumour spheroids or tumour cells on air-liquid interfaces is a more holistic approach and mimics the complete tumour, however it requires a more substantial (mostly surgical) specimen, which excludes the use for neo-adjuvant strategies.
ii. Co-culture systems of PDO with separately sampled cells of the microenvironment benefit from pre-established PDO culture protocols. These models have the advantage that specific cellular components of the microenvironment can be addressed, and only small tumour specimens are necessary for PDO generation.

Since the need for these complex PDO models and the strategies to address the microenvironment are rather new, there are some promising reports indicating that establishing complex PDO models harbouring different cellular compartments are feasible and representative for the original tumour (53, 56–59)

The advances of organoid research in both cancer and inflammation (non-malignant) gave rise to potential model systems, which, for instance, could depict the transition from chronic inflammation to inflammatory-driven carcinogenesis.

STANDARDISATION REMAINS THE MAJOR CHALLENGE FOR *IN VITRO* INFLAMMATION MODELS

As outlined, enormous advances in the stem cell and organoid field have emerged and their potential in translational research, and even healthcare, is obvious; however, current limitations of organoids remain an important caveat (**Figure 2B**).

A general limitation of organoid derivation is the high variability of the phenotypes that they can produce. Organoid and stem cell culture systems and their molecular outcome are critically dependent on the quality and properties of the sampled

A

	Cell line	Cell line co-cultures	iPS	GEM	MDO	PDO
Primary culture	🔴	🟢	🟢	🟢	🟢	🟢
Preinvasive cancer model	🔴	🔴	🟢	🟢	🟢	🟢
Patient-derived material	🟡	🟢	🔴	🔴	🔴	🟢
Costs	🟢	🟢	🟡	🔴	🟡	🟡
Success rate	🟢	🟢	🔴	🟡	🟡	🟡
High-throughput screening	🟢	🟢	🟢	🔴	🟡	🟡
Time	🟢	🟢	🟡	🔴	🟡	🟡

B

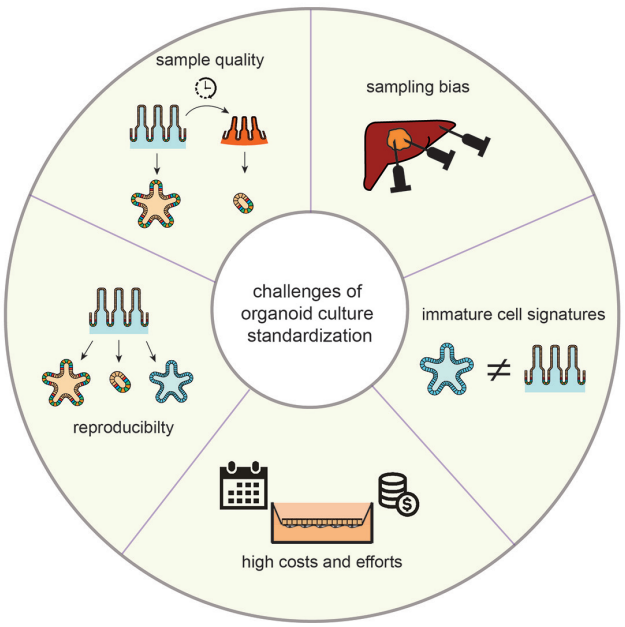

FIGURE 2 | **(A)** Properties of the discussed models for translational research; GEM, genetically engineered mouse model; MDO, murine-derived organoid; iPS, induced pluripotential stem cell; PDO, patient-derived organoid; definition of colours: Green - possible/cheap (costs)/easy (success rate)/1–2 weeks (time); Yellow: medium (costs)/medium (success rate)/1–2 months (time); orange – 1–6 months (time); red - difficult/expansive (costs)/low (success rate)/over 6 months (time). **(B)** Overview over the proposed challenges in the standardisation of organoid culturing systems.

tissue. Variations in the pre-processing phase heavily interfere with immune signatures of these samples. Moreover, inflamed tissue is more likely to undergo cell death, might have different nutrient and culturing needs and thus the recovery rate of vital organoids from inflamed tissue is significantly lower (60). Whilst inflammatory signatures *ex vivo* are similar to their *in vivo* origin (9), particularly complex diseases such as IBD are characterised by a diversity of inflammatory flavours. Furthermore, biopsies are reflecting the biology of the sampled region, and therefore stem cells/organoids should be harvested from different regions. When using iPSCs, variations exist in organoids depending on the genetic background of the individual and the culture protocol used by the lab. Isogenic lines generated *via* gene-editing approaches can limit this variability in regard to genetic differences.

Because organoids from iPSCs are formed through differentiation of a homogeneous population, tissue-specific cell types and their microenvironment must be newly created. This challenges the use of iPSCs and despite significant similarities in structure and function between organoid and adult tissue, organoids often retain immature characteristics, making them more similar to foetal tissue. In an example of iPSC-derived intestinal organoid, this limitation could be resolved by either co-culturing it with T cells providing a more realistic growth niche (61), engineering of more sophisticated 3D scaffolds to improve organoid architecture and thus increase the similarity to the original tissue *in vivo* (62, 63), or

in vivo transplantation, which resulted in mature intestinal epithelium with preserved intestinal stem cell niches, crypt/villus architecture and a laminated human mesenchyme, both supported by mouse vascular ingrowth (64). Apart from immature characteristics, organoids often show a limited lifespan once a certain size is reached. Due to the lack of diffusion, cells are not supplied with sufficient nutrients to support continued development. The use of bioreactors could improve the nutrient supply. Ideally, organoids are engineered towards the induction of endothelium development resulting in vascularization as shown recently (41). Another strategy would be to integrate endothelial cells, or their progenitors, during organoid development, and to include bioprinting methods to design 3D-scaffolds for the endothelial cells (65, 66). However, the original material tissue of iPSC-derived organoids is usually not inflamed, e.g. skin fibroblasts, and have been reprogrammed and cultured for many weeks to months in the absence of an inflammatory milieu. For the study of complex, multifactorial inflammation, iPSC-derived systems are therefore not the first choice in terms of a model. Vice versa, iPSC-derived organoids might be excellent tools to study mainly genetically driven inflammatory disorders, such as monogenic IBD.

Another important challenge is the magnitude of effort, time and expenses spent on organoid cultures. Whilst rather simple protocols are available for conventional organoid cultures at manageable costs, differentiation protocols include either expensive factors or culturing media or require conditioned media, which need to be produced by feeder cell lines. Co-culturing systems often include 2D models, which require much higher culturing efforts and time. Adding to this challenge, the generation of iPSC and their differentiation into specific tissue often require several weeks of intense culturing effort, which increases the risk of adverse events, e.g. contamination or undesired differentiation.

To combat the aforementioned issues, close collaboration of academic research and high-tech industrial partners may be a promising strategy to overcome the infrastructural challenges. Whilst academia can provide problem-derived ideas and a hypothesis-driven vision of novel disease models, industry can deliver the necessary technology, capacities for large-scale production and standardisation. Setting up these collaborative infrastructures are necessary and can foster future advances of stem cell technology.

CONCLUSIONS AND FUTURE PERSPECTIVES

Organoids have been shown to keep key multicellular, anatomical and to some degree even functional hallmarks of real organs. In order to exploit these improved disease models, the application of high-throughput analysis techniques and large-scale perturbation tools to organoids is required. Single-cell multi-omics (19, 20) and imaging technologies can provide insight into underlying pathological mechanisms at different regulatory molecular layers. Physiological interactions of different organs could be modelled by using microfluidics and organ-on-a-chip technologies to study key systemic interactions in diseases. The combination of advanced organoid models and these techniques might even enable to understand the early molecular mechanisms that cause cells to deviate from a healthy to a disease trajectory (67). This might lead to the detection of biomarkers for the prodromal disease state and the identification of new drug targets to intercept diseases before manifestation of symptoms.

One of the visions of stem cell research is the development of stem-cell-derived regenerative tissue for engraftment and transplantation purposes. Recently, a group successfully engrafted human iPSC-derived kidney organoids into immunodeficient NOD/SCID mice, which reached a higher degree of maturation compared to *in vitro* kidney organoids but still markedly immature compared to the neighbouring mouse kidney tissue (68). Besides tissue repair, this technology can be a promising vehicle for targeted gene therapy, especially for monogenic disorders. The advances in CRISPR/Cas9 technology already allow selective genome editing with a reduced number of off-target effects by using engineered secondary RNA structures (69) or by implementing prime editing guide RNA that specifies the target site and encodes the desired edit (70). However, only complete and definite exclusion of any off-target DNA alterations are acceptable for transplantation purposes.

It will be of great importance to implement organoids as a platform for screening and testing personalised medicine treatments, as they are cultures of primary patient material. Whilst this is already possible for cancer (54), it will in the future also be possible for inflammatory disorders that affect epithelial tissues. We expect implementation of these systems in drug discovery, therapy guidance and tissue regenerative medicine. Therefore, joint efforts of academia and industrial partners are mandatory to surpass challenges in regard to safety and reliability.

AUTHOR CONTRIBUTIONS

All authors contributed to the discussion at the symposium workshop. The manuscript was conceptualised by FT, CK, AA, SI, PR, and PS, whilst FT coordinated the manuscript writing. The figures and tables were designed by FT, AA, and PS. All authors contributed to the article and approved the submitted version.

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REVIEW
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Milestones in Personalized Medicine in Pemphigus and Pemphigoid

Katja Bieber[1], *Khalaf Kridin*[1], *Shirin Emtenani*[1], *Katharina Boch*[2], *Enno Schmidt*[1,2] *and Ralf J. Ludwig*[1,2]*

[1] Lübeck Institute of Experimental Dermatology and Center for Research on Inflammation of the Skin, University of Lübeck, Lübeck, Germany, [2] Department of Dermatology and Center for Research on Inflammation of the Skin, University of Lübeck, Lübeck, Germany

Pemphigus and pemphigoid diseases are autoimmune bullous diseases characterized and caused by autoantibodies targeting adhesion molecules in the skin and/or mucous membranes. Personalized medicine is a new medical model that separates patients into different groups and aims to tailor medical decisions, practices, and interventions based on the individual patient`s predicted response or risk factors. An important milestone in personalized medicine in pemphigus and pemphigoid was achieved by verifying the autoimmune pathogenesis underlying these diseases, as well as by identifying and cloning several pemphigus/pemphigoid autoantigens. The latter has become the basis of the current, molecular-based diagnosis that allows the differentiation of about a dozen pemphigus and pemphigoid entities. The importance of autoantigen-identification in pemphigus/pemphigoid is further highlighted by the emergence of autoantigen-specific B cell depleting strategies. To achieve this goal, the chimeric antigen receptor (CAR) T cell technology, which is used for the treatment of certain hematological malignancies, was adopted, by generating chimeric autoantigen receptor (CAAR) T cells. In addition to these more basic science-driven milestones in personalized medicine in pemphigus and pemphigoid, careful clinical observation and epidemiology are again contributing to personalized medicine. The identification of clearly distinct clinical phenotypes in pemphigoid like the non-inflammatory and gliptin-associated bullous pemphigoid embodies a prominent instance of the latter. We here review these exciting developments in basic, translational, clinical, and epidemiological research in pemphigus and pemphigoid. Overall, we hereby aim to attract more researchers and clinicians to this highly interesting and dynamic field of research.

Keywords: precision medicine, pemphigus, pemphigoid, diagnosis, treatment

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Roberto Maglie,
University of Florence, Italy

***Correspondence:**
Ralf J. Ludwig
ralf.ludwig@uksh.de

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PEMPHIGUS AND PEMPHIGOID

Pemphigus and pemphigoid are autoimmune diseases of the skin and/or mucous membranes characterized and caused by autoantibodies targeting structural proteins (1). In individual patients, the specific pemphigus or pemphigoid disease is diagnosed based on the clinical presentation, the detection of tissue-bound autoantibodies and the autoantibody specificity (2–4). Albeit rare, pemphigus and pemphigoid diseases impose a major disease burden with a high unmet medical

need (5, 6). The clinical hallmark of pemphigus and pemphigoid is (muco)-cutaneous blistering, which occurs intradermal in pemphigus and subepidermal in pemphigoid. In both diseases, autoantibodies are generated in a CD4-dependent fashion. As a general principle, blistering occurs directly as a result of autoantibody binding to the target antigens and *via* complement-independent mechanisms in pemphigus, whereas blistering in pemphigoid usually depends on the activation of innate immune responses through the Fc-portion of the autoantibodies (7, 8). Pemphigus is currently treated with high-dose corticosteroids and the anti-CD20 antibody rituximab, which achieves complete remissions in 80% of the patients (9). The most effective treatment of bullous pemphigoid (BP), by far the most common pemphigoid disease, is long-term application of superpotent topical or oral corticosteroids (10). Epidermolysis bullosa acquisita (EBA) is another pemphigoid disease characterized by a chronic course and is often more refractory to treatment as compared to BP. The main challenges in treating pemphigus are the relative long time needed to induce remissions, high rate of adverse events, and relapse after stopping treatment (11). In BP, relapses after cessation of treatment are the main challenge, as these leads to prolonged treatment with corticosteroids, which are partially responsible for the increased morbidity and mortality of the patients (12).

The current research on pemphigus and pemphigoid diseases is, in our opinion, based on the landmark discovery by Walter Lever in 1953, who, for the first time, clearly differentiated between pemphigus and pemphigoid diseases, mainly based on the histology of skin affected by either one of the diseases (13). This differentiation between pemphigus and pemphigoid based on lesional histopathology promoted tailoring specific treatments for patients with autoimmune bullous diseases (AIBDs), as patients with pemphigus necessitated more aggressive immunospuressive therapy as compared to their counterparts with BP. Subsequently, further milestones in personalized medicine in pemphigus and pemphigoid were made (**Figure 1**):

1. Identification of distinct patterns of autoantibody deposits in the skin
2. Discovery of unique autoantigens in distinct pemphigus and pemphigoid diseases
3. Defining pemphigus and pemphigoid as autoimmune diseases
4. Establishing the current, molecular-based diagnosis of pemphigus and pemphigoid
5. Exploring the functionally relevant molecules and pathways by the use of complex model systems
6. Defining unique pemphigus and pemphigoid variants based on epidemiology
7. Moving towards personalized treatment, selectively targeting specific, autoreactive B cells

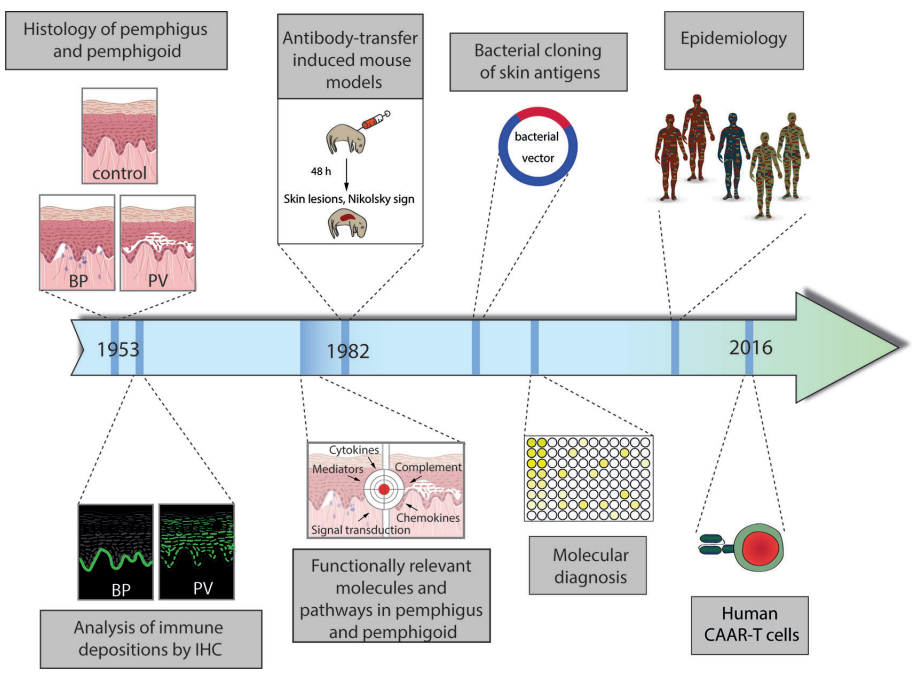

FIGURE 1 | Schematic diagram of the milestones in personalized medicine in pemphigus and pemphigoid. (1) Distinction of pemphigus and pemphigoid based on clinical observation and histology of lesional skin. (2) Identification of distinct binding patterns of autoantibodies in pemphigus (exemplified by pemphigus vulgaris, PV) and pemphigoid (exemplified by bullous pemphigoid, BP). (3) Proof of the autoimmune pathogenesis of pemphigus and pemphigoid by transfer of patient IgG into mice with the subsequent development of pemphigus. (4) Identification and cloning of autoantigens. (5) Establishment of the current, molecular-based diagnostics of pemphigus and pemphigoid. (6) Identification of unique subgroups by careful clinical observation and epidemiology. (6) Individualized treatment by the use of chimeric autoantigen receptor (CAAR) T cells. More details are given in the text.

These milestones in personalized medicine in pemphigus and pemphigoid are presented in detail in the following sections.

HISTOLOGICAL DIFFERENTIATION BETWEEN PEMPHIGUS AND PEMPHIGOID

The term "pemphigus" was used as descriptive terms for skin diseases characterized by blisters since Hippocrates (460-370 B.C.) who described different types of fever associated with blistering as "pemphigus fever". However, the term pemphigus in its present meaning was coined by Dr. Wichman in 1791 when describing a case of pemphigus (in today's understanding). Thereafter, "pemphigus" was used as a synonym for any vesicular or bullous disease. This led to the emerge of several different types of "pemphigus" (14). In 1860, Dr. Hebra reinstated the concept established by Wichman, stating that "pemphigus" is always a chronic disease. Thus, all pemphigus and pemphigoid diseases were subsumed under the term "pemphigus" (15). Based on the prognosis, two different forms of "pemphigus" were differentiated: Malignant and benign pemphigus. In 1953, Walter Lever published his landmark histological observations where he distinguished between pemphigus, characterized by intraepidermal blistering, and BP, characterized by subepidermal blistering (13) (**Figure 2**). Taking the clinical presentation into account, he also coined the term mucous membrane pemphigoid (MMP) that is characterized with a histology similar to BP but with blistering at mucosal sites (13). Thus, Walter Levers' careful clinical and histological

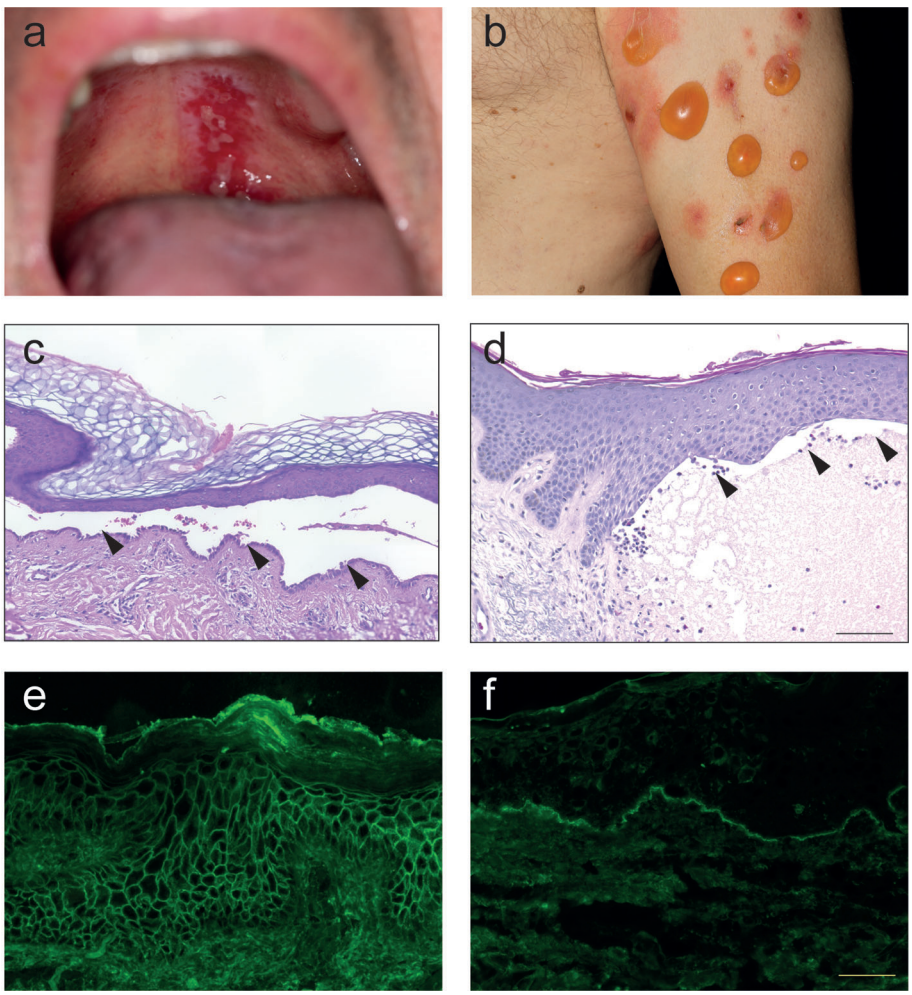

FIGURE 2 | Clinical, histological and immunological features of pemphigus and pemphigoid. **(A)** Clinical presentation of pemphigus vulgaris with erosions at the upper palate. **(B)** Clinical presentation of bullous pemphigoid with blistering at erythematous or otherwise apparently healthy skin. **(C)** Lesional histopathology of a patient with pemphigus vulgaris showing suprabasal splitting with some acantholysis and the typical "row of tombstones" (arrows). **(D)** In a lesional biopsy of a patient with bullous pemphigoid, subepidermal splitting. In this case, interestingly, the dermal infiltrate is only marginally present. **(E)** Tissue-bound IgG in a perilesional skin biopsy of a pemphigus vulgaris patient, showing IgG deposits in a honeycomb-like pattern within the epidermis. **(F)** Tissue-bound IgG in a perilesional skin biopsy of a bullous pemphigoid patient, showing linear IgG deposits along the dermal-epidermal junction.

observations still hold true and are a good example of how thorough clinical observations allow landmark discoveries.

IDENTIFICATION OF UNIQUE AUTOANTIBODY DEPOSITS IN PEMPHIGUS AND PEMPHIGOID

About two decades after the discovery of Dr. Lever, IgG deposits were noted in patients with pemphigus (16) and pemphigoid (17). In pemphigus, immunoglobulin (Ig) deposits were found to be located at the surface of the keratinocytes. Thus, the term honeycomb-like fluorescence observed within the epidermis has been coined for this particular staining pattern (**Figure 2**). In pemphigoid diseases, linear Ig/complement (C) deposits along the dermal-epidermal junction are observed. For this, staining pattern the term "linear" Ig/C deposition has been established (**Figure 2**). More recently, unique patterns of IgG deposits have been observed in pemphigoid diseases. In contrast to all other pemphigoid diseases where the IgG deposits show a "n-serrated" pattern, the pemphigoid disease EBA displays an "u-serrated" pattern of both IgG and IgA (18–20). The identification of these patterns in direct immunofluorescence (IF) microscopy is a learnable skill (21), which is essential for the diagnosis of EBA because up to 60% of cases are seronegative (22). The observation of these unique staining patterns in pemphigus and pemphigoid (as well as in EBA) supported the histopathological observation in 1953 that pemphigus and pemphigoid are distinct diseases.

DEFINING PEMPHIGUS AND PEMPHIGOID AS AUTOIMMUNE DISEASES

The deposition of IgG in the skin suggested that pemphigus and pemphigoid may be caused by an immune response to self-antigens. Yet, direct proof of the autoimmune pathogenesis of pemphigus and pemphigoid were missing. According to the revised Witebsky's postulates, such direct proof is the induction of the disease in experimental animals by transfer of patient serum or IgG (23). The autoimmune nature of pemphigus was demonstrated by Grant Anhalt and colleagues in 1982, when they induced skin blistering and erosions, accompanied by the histologic, ultrastructural, and immunological findings found in pemphigus patients, by transfer of pemphigus patient IgG into neonatal Balb/c mice (24). Attempts to reproduce the pemphigoid disease BP by transfer of patient IgG into mice failed because the transferred IgG did not bind to the skin of the mice (25). Several years later, in 1993, when the autoantigens in BP had been defined (BP180 and BP230), and the lack of cross-reactivity of human autoantibodies with murine BP180 had been demonstrated, Zhiou Liu and colleagues were able to induce experimental BP in mice by the transfer of rabbit anti-mouse BP180 IgG (26). Collectively, the defining of pemphigus and (later) pemphigoid as autoimmune diseases, and the demonstration of distinct clinical, histological and immunological features in experimental pemphigus and pemphigoid triggered the search for the autoantigens. Subsequently, the pathogenic relevance of several autoantigens and their corresponding antibodies has been demonstrated in different mouse models for pemphigus foliaceus (PF), paraneopalastic pemphigus (PNP), MMP, and EBA.

IDENTIFICATION OF DISTINCT AUTOANTIGENS IN PEMPHIGUS AND PEMPHIGOID

Identification of Ig deposits in the skin, as well as the proof of the autoimmune pathogenesis of pemphigus and pemphigoid initiated the search for putative autoantigens, which is still ongoing; i.e., in the case of anti-p200 pemphigoid (27). Using patient autoantibodies, and monoclonal antibodies against type VII collagen (COL7), COL7 was identified as the autoantigen in EBA, as early as in 1988 (28). In 1990, BP180 was cloned human from a keratinocyte library (29). One year later, both, desmoglein (Dsg) 1 and 3 were cloned and identified as the autoantigens in PF and pemphigus vulgaris (PV) (30–32). Subsequently, additional autoantigens in pemphigus and pemphigoid were cloned and/or identified (**Table 1**).

MOLECULAR-BASED MODERN DIAGNOSIS OF PEMPHIGUS AND PEMPHIGOID

Definition of distinct autoantigens in pemphigus and pemphigoid diseases enabled the development of the currently used, molecular-based diagnosis of pemphigus and pemphigoid (37). If clinically suspected, the detection of tissue-bound autoantibodies (or C3) in a perilesional skin (or mucosal) biopsy is the gold standard for the diagnosis of pemphigus and pemphigoid. Depending on the location of the Ig or C3 deposits, pemphigus (deposits in the intercellular space, also termed honeycomb pattern), or pemphigoid (linear staining along the dermal-epidermal junction) are differentiated. EBA can be further differentiated based on the serration pattern (19). This pattern analysis is a learnable and important skill because EBA may be seronegative in 60% of the cases (21, 22).

If the diagnosis cannot be established based on the direct IF microscopy, indirect IF microscopy using different organ substrates, most frequently monkey esophagus and human salt-split skin, can further differentiate between the different pemphigus and pemphigoid diseases (37). Other less frequently utilized substrates are rat bladder for PNP and normal oral mucosa for MMP (38). While monkey esophagus is useful in the detection of circulating pemphigus-related autoantibodies, pemphigoid autoantibodies better bind to salt split skin. Of note, if a linear deposit of patient autoantibodies is observed at the roof of the blister of the artificially split skin, BP (or MMP in patients with predominant mucosal involvement) is diagnosed

TABLE 1 | Autoantigens in pemphigus and pemphigoid.

	Disease	Main target antigen(s)	Main isotype(s)
Pemphigus	**Pemphigus vulgaris (PV)**	Dsg3 in mucosal PV, Dsg 1 and 3 in muco-cutaneous PV	IgG
	Pemphigus foliaceus (PF)	Dsg 1	IgG
	Paraneoplastic pemphigus (PNP)	Envoplakin, periplacin, Dsg 1/3, BP180, and others	IgG
	IgA pemphigus	Dsg 1/3, Dsc 1-3	IgA
	Endemic pemphigus foliaceus	Dsg 1	IgG
	Herpetiform pemphigus	Dsg 1	IgG
Pemphigoid	**Bullous pemphigoid (BP)**	BP180-NC16A, BP230	IgG/IgE
	Mucous membrane pemphigoid (MMP)	BP180, BP230, laminin-332, $\alpha4\beta6$ integrin, laminin-331, COL7	IgG
	Pemphigoid gestationis (PG)	BP180-NC16A	IgG
	Linear IgA disease (LAD)	LAD-1	IgA
	Epidermolysis bullosa acquisita (EBA)	COL7	IgG/IgA
	Anti-.p200 pemphigoid	Laminin $\gamma1$ (non-pathogenic autoreactivity)	IgG
	Lichen planus pemphigoides	BP180-NC16A, BP230	IgG

Please note that pemphigus may also be induced by non-Dsg autoantibodies (33, 34). However, in >95% of pemphigus vulgaris/foliaceus patients, anti-Dsg autoantibodies are present (35, 36). Thus, non-Dsg autoantibodies were not included in this table. Dsg, desmoglein; Dsc, desmocollin; COL7, type VII collagen; LAD-1, linear IgA disease antigen-1 (soluble ectodomain of BP180).

because its autoantigens (BP180 and BP230) are expressed at the blister roof. By contrast, laminin-332, COL7 and p200 are expressed at the blister floor. Hence, binding of patient autoantibodies to the blister floor in indirect IF microscopy on salt split skin requires further differentiation between MMP, EBA, and anti-p200 pemphigoid. The latter, as well as (semi)-quantitative determination of circulating autoantibody concentrations, can be achieved by detection of specific autoantibodies. This can be achieved using the recombinant immunodominant domains of the target antigens, i.e., Dsg1, Dsg3, envoplakin, BP180, BP230, laminin 332, and COL7 in commercial ELISA systems or biochip mosaics. In addition, specialized laboratories have established techniques (mainly Western blotting or immunoprecipitation) for the detection of autoantibodies against p200, selected chains of laminin-332, the ectodomain of BP180, or rare autoantigens. Thus, this molecular-based modern diagnosis of pemphigus and pemphigoid allows (in most cases) to diagnose individual pemphigus and pemphigoid diseases. As their treatment and prognosis greatly differs (2, 3), this allows to select the appropriate treatment for each patient. In addition to their use in diagnosis, longitudinal monitoring allows for early detection of relapses because circulating autoantibody concentrations correlate intraindividually with disease activity (39–41). Molecular characterization of some AIBD, however, is still possible only in highly specialized academic centers. A possible consequence is the delay experienced by patients in receiving the right diagnosis and optimal treatment.

A more recent development in the personalized management of pemphigoid is the identification of biomarkers other than the autoantibodies that allow to predict treatment response and/or relapse (42). In brief, persistence of elevated eosinophil cationic protein (ECP) in patients with BP was associated with relapse (43). In addition to persistence of elevated ECP levels, the presence of anti-COL7 autoantibodies (the autoantibody in EBA) (44), and increased CXCL10 serum levels (45) are also predictors of relapse in patients with BP. Relative to non-autoreactive B cells, autoreactive B cells of patients with PV showed overexpression of genes encoding for IL-1β, IL-23p19, and IL-12p35 pro-inflammatory cytokines and the IRF5 transcription factor. Relative to patients with active pemphigus, those experiencing complete remission following rituximab displayed under-expression of IL-1β and the CD27 memory marker genes (46).

Efforts were extensively made to establish a personalized approach to optimize management of patients with pemphigus. That is, to predict patients predisposed to early relapses under rituximab who may benefit from maintenance rituximab infusion at month 6. Increased severity score at baseline and increased levels of anti-Dsg1 and anti-Dsg3 three months following the first infusion were found to independently project post-rituximab early relapse, thus warranting to consider a maintenance dose of rituximab after 6 months (47).

FUNCTIONALLY RELEVANT MOLECULES AND PATHWAYS IN PEMPHIGUS AND PEMPHIGOID

Employing keratinocyte cultures, *ex vivo* skin models, and the above-mentioned mouse models (48, 49), several disease pathways have been identified that provide the base for valuable novel therapeutic approaches. Dsgs are transmembrane desmosomal cadherin-like glycoproteins which function to maintain tissue integrity and facilitate cell-cell adhesion. IgG autoantibodies targeting Dsg3 and Dsg1 play the main etiopathogenetic role in the development of PV and PF, respectively. In pemphigus, monovalent fragments of anti-Dsg antibodies that lack the Fc portion are sufficient to cause acantholysis *in vitro* and *in vivo* (50). The exact sequence of events in anti-Dsg antibody-mediated acantholysis has not yet been fully understood. Three major events following the binding of anti-Dsg IgG have been described: (i) direct interference with Dsg transinteraction, a phenomenon termed steric hindrance, (ii) remodeling of Dsg expression on the cell surface leading to internalization and depletion of Dsg from the cell membrane, and (iii) signaling events that impair cytoskeletal architecture (3, 4). These mechanisms do not apply equally for Dsg1 IgG- and Dsg3

IgG-binding. Upon targeting of Dsg1, Ca^{2+} influx is induced and the ERK pathway is activated. In contrast, after binding of Dsg3-specific IgG, signaling *via* p38MAPK occurs in the epidermis but not in mucosal tissues, and SRC family of protein tyrosine kinases and EGFR pathways are activated (51, 52). Current data also strongly suggest that, in addition to Dsg1/3 autoantibodies, non-Dsg antibodies, as well as soluble Fas ligand contribute to the pemphigus phenotype (53–56).

In contrast to pemphigus disorders, in pemphigoid diseases, FcγR-mediated effects are pivotal for blister formation, and over the last decade several disease pathways and key molecules with functional relevance in these diseases have been described including several signaling molecules, leukotriene B4 (LTB4), and IL-17 (57–60). Furthermore, complement activation at the dermal-epidermal junction is generally accepted to be a cornerstone in recruiting neutrophils, eosinophils, and macrophages to this site (61). Of note, subtle differences in the impact of complement activation emerged between different pemphigoid diseases as well as between clinical variants of BP. In contrast, acantholysis in pemphigus appears to develop independently of complement activation although staining of C3 in the epithelium/epidermis is a diagnostic hall mark (3). Exploring these differences may uncover patient and diseases subgroups that can benefit from therapeutic interventions in targeting complement components. About 80% of BP patients reveal C3c deposition along the dermal-epidermal junction in perilesional biopsies. In patients with C3c deposition, anti-BP180 NC16A IgG serum levels were significantly higher and patients without blisters had significantly less C3c deposits along the dermal-epidermal junction (62). While no relation between the extent of skin lesions and C3c staining in the skin of patients was found, the complement activation capacity of autoantibodies in the *ex vivo* complement fixation assay correlated with diseases activity as measured by the Bullous Pemphigoid Disease Area Index (BPDAI) (63). Further support for the relevance of complement activation in BP comes from the finding of elevated levels of C3a in the serum of BP patients and the positive correlation of serum C3a levels with both anti-BP180 NC16A IgG and soluble CD46, a crucial complement regulatory protein in the complement activation (64). However, plasma concentrations of C3a, C4a, as well as C5a are not different between BP patients and age/sex matched controls. Furthermore, the plasma levels of these three complement components remain constant when evaluated in flares and in remission of BP (65). In the neonatal BP mouse model, where blisters typically develop 24–48 h after injection of rabbit IgG against the NC15A domain of BP180, the blistering phenotype was completely dependent on complement activation at the dermal-epidermal junction (66, 67). The same complement dependency was observed in a humanized mouse model of BP in which the human NC16A domain replaced the homologous murine NC15A region (68). In contrast, in another humanized mouse model of BP, in which the entire BP180 molecule had been replaced by the human protein, injection of polyclonal F(ab')2 anti-NC16A IgG or non-complement-activating anti-NC16A IgG4 led to blister formation (69, 70). In line, Dainichi et al. reported on two BP patients without C3 deposits in the skin and IgG4 autoantibodies as main subclass that were unable to elicit complement activation *ex vivo* (71). Additional observations in this model with C3-deficient animals that were susceptible to the pathogenic effect of anti-BP180 IgG and recently, in a BP model in adult mice where transfer of anti-BP180 NC15A IgG in C5aR1-deficient mice led to a reduction of skin lesions by 50% (70, 72, 73) pointed to complement-independent mechanisms in BP pathophysiology. Interestingly, similar to the BP neonatal mouse model, in adult mouse models of EBA and anti-laminin 332 MMP, complement activation appeared as prerequisite for a blistering phenotype (74–76). Unravelling the complex scenario of complement activation in pemphigoid disorders will certainly help to identify patient subpopulations and to tailor more specific and safe treatments for these diseases. The dose-dependent inhibition of the BP IgG-induced C3 deposition at the dermal-epidermal junction in the *in vitro* complement fixation on cryosections of human skin by (i) the anti-C1s antibody TNT003, (ii) the low-molecular-weight heparin tinzaparin sodium, and (iii) the dual C5/LTB4 antagonist coversin, all of the which disturbing the normal activity of complement pathway (65, 77, 78) directs us to further explore complement inhibition as valuable therapeutic target in BP.

EPIDEMIOLOGY DEFINES UNIQUE VARIATIONS WITHIN SEVERAL PEMPHIGUS AND PEMPHIGOID DISEASES

As its name indicates, PV is the most prevalent subtype of pemphigus, comprising up to 70% of all cases (79). PV is typified by a variable geographic and ethnic distribution, with annual incidence rates ranging between 0.8 and 16.1 cases per million population in different regions (80). Congruently, a predisposition for developing pemphigus was reported in some ethnic groups, namely, Ashkenazi Jews and individuals of Mediterranean origin (80). In a recent population-based study, the incidence of PV was 3.6-fold increased among Jews as compared to Arabs in Israel (81). In a retrospective study conducted in Connecticut, the US, the annual incidence of PV was almost eight-fold higher among people of Jewish ancestry than among those belonging to other ethnic groups (82). These epidemiological observations have been strongly substantiated by genetic studies disclosing an association of several HLA-class II genes, HLA-DRB1*04, and HLA-A*10, with the occurrence of PV among Ashkenazi Jews (83–85). Subsequently, a polymorphic variant in *ST18* gene was associated with PV in Jewish and Egyptian but not in German patients (86). Hence, despite an underlying genetic pre-disposition in PV, the disease may manifest also in the absence of certain genetic predisposing factors. Collectively, this points towards a polygenic genetic risk to develop PV, as well as points towards the environment as a potential driver of disease pathogenesis, as reported for other inflammatory diseases (87).

Sporadic PF is an uncommon disease constituting 20%–30% of all pemphigus cases. Its estimated annual incidence in Europe and the United States is less than one case per million population (80). HLA-DRB1*04 was associated with increased risk for sporadic PF in Brazilian, Italian, French, and Dutch populations (88, 89). An association with HLA-DRB1*0101 was found among patients with sporadic PF originating from Mexico (90). Nevertheless, no ethnic predilection in the occurrence of PF was noted in Israel, as the adjusted incidence rate of the disease was comparable between Jews and Arabs (81). Apart from sporadic cases, endemic subtypes of PF have been described in Brazil (*folgo selvagem)*, Colombia, and Tunisia (80). Although patients with endemic PF are clinically, histologically, and immunologically indistinguishable from those with sporadic PF (80, 91), the former is characterized by a patterned geographic distribution, familial predisposition, and younger age of presentation (92, 93).

BP is the most common subepidermal AIBDs worldwide. BP characteristically affects the elderly and is seen mainly among patients older than 75 years. While the general annual incidence of BP has been reported to range between 2.4 and 23.0 cases per million population, it rises exponentially to 312 cases per million population in individuals older than 80 years (94). Several lines of evidence accumulated to suggest a notable increasing incidence of BP by 1.9 to 4.3 folds over the past two decades (95). Several putative interpretations were postulated to account for this observation, the most plausible of which is the growing exposure to certain medications implicated with the induction of BP (95). Dipeptidyl-peptidase IV inhibitors (DPP4i), also known as gliptins, a recently introduced anti-diabetic class of oral medications, emerged as a potential trigger of BP (96–98). It is yet to be decisively determined whether patients with DPP4i-associated BP follow a distinct clinical and immunological profile. While European studies did not depict distinct features for these patients distinguishing them from typical BP, DPP4i-associated BP patients originating from Japan were more likely to present with non-inflammatory BP and to target the immunodominant domain of BP180 antigen (NC16A) less frequently (99–101). Thus, careful clinical observation has led to the identification of a unique clinical presentation of BP. Given that the initial observations of unique immunological and genetic features of DPP4i-asscociated BP can be confirmed, this would allow to treat the patients according to the underlying, disease-promoting pathways. In the case of DPP4i-associated BP, this is relatively easy because BP usually clears after stopping DPP4i treatment.

CAAR T CELLS AS A POTENTIAL AND PERSONALIZED CURE OF PEMPHIGUS AND PEMPHIGOID

The most recent advance towards a personalized treatment approach is the development of chimeric autoantigen receptor (CAAR) T cells for the treatment of pemphigus (102). Based on the breakthrough discovery of chimeric antigen receptor (CAR) T cells for the treatment of hematologic malignancies (103), Aimee Payne and colleagues developed a recombinant T cell receptor by fusing the autoantigen in PV (Dsg3) to CD137-CD3ζ. These Dsg3 CAAR T cells exhibited specific cytotoxicity against B/plasma cells expressing the B cell receptor specific for Dsg3. Next, experimental pemphigus was induced in mice by transfer of Dsg3-hybridoma cell lines. When mice were additionally injected with Dsg3 CAAR T cells, they were protected from induction of experimental pemphigus (102). Subsequent work from the same group expanded the work to additional autoantigens in pemphigus, namely, Dsg1 (104). Currently, a phase I clinical trial is conducted on autologous Dsg3 CAAR T cells in mucosal PV (https://cabalettabio.com/clinical-trials/, accessed May 31, 2020). By selectively targeting autoreactive B cells, using the CARR T cell technology, a highly personalized treatment approach for PV is currently under development (105). Overall, given successful completion of this (and subsequent) clinical trials, a new era of managing B cell-driven autoimmune diseases (106) will emerge.

FUTURE PERSPECTIVES

The emergence of treatments selectively targeting autoreactive B cells, e.g., by CAAR T cells is expected to significantly change the treatment of pemphigus. It is highly intriguing to estimate whether CAAR T cells the same potential additionally in pemphigoid diseases. Alternatively, in another approach, immunization of PV patients with Dsg3-coated nanoparticles may specifically suppress autoimmunity against Dsg3 and is currently performed in a phase I clinical trial in PV with Dsg3. Targeting IL-17 and eotaxin is currently being assessed as a potential therapeutic approach in BP (107). The success of these treatments will, however, depend on an expansion of the molecular diagnosis that will allow to precisely define the autoimmune response in individual patients. In parallel, molecular diagnosis is also expected to help define distinct (sub)-groups of pemphigus and pemphigoid diseases that most likely will be more and more based on molecular signatures. In the long term, one may envision that curative and safe treatments for pemphigus and pemphigoid will be available.

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Expansion of Monocytic Myeloid-Derived Suppressor Cells in Patients Under Hemodialysis Might Lead to Cardiovascular and Cerebrovascular Events

Yan-Fang Xing[1†], Jia-Rong Cai[2†], Jun-Jian Qin[1†], Wen-Ying Zhou[3], Can-Ming Li[4] and Xing Li[5*]*

[1] Department of Nephrology, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [2] Department of Urology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [3] Department of Central Laboratory, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [4] Department of Nephrology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [5] Department of Medical Oncology and Guangdong Key Laboratory of Liver Disease Research, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

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Ralf J. Ludwig,
University of Lübeck, Germany

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Robert Campbell,
The University of Utah, United States
Unni Krishna SRL Samavedam,
University of Cincinnati, United States

***Correspondence:**
Xing Li
lixing9@mail.sysu.edu.cn
Yan-Fang Xing
2574410512@qq.com

[†]These authors have contributed equally to this work

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Background: The specific mechanism of cardiovascular and cerebrovascular vasculopathy in the context of end-stage renal disease has not been elucidated. In the present study, we investigated the clinical impact of myeloid-derived suppressor cells (MDSCs) on hemodialysis patients and their mechanism of action.

Methods: MDSCs were tested among 104 patients undergoing hemodialysis and their association with overall survival (OS) and cardiovascular and cerebrovascular events was determined.

Results: Hemodialysis patients presented a significantly higher level of monocytic MDSCs (M-MDSCs) compared to healthy controls. M-MDSC were tested 3 months after first testing among 103 hemodialysis patients, with one patient not retested due to early death. The repeated results of M-MDSC levels were consistent with the initial results. Patients with persistent high level of M-MDSCs presented decreased OS, as well as increased stroke and acute heart failure events. As illustrated by multivariate Cox regression, M-MDSC was an independent predictor for OS and stroke events of hemodialysis patients. T cell proliferations were significantly abrogated by hemodialysis-related M-MDSCs in a dose-dependent manner. Besides, M-MDSCs presented higher levels of CXCR4 and VLA-4 compared to monocytes, which indicated their enhanced capability to be recruited to atherosclerotic lesions. The expression of arginase I and activity of arginase was also significantly raised in hemodialysis-related M-MDSCs. Human coronary arterial endothelial cells (HCAECs) presented increased capability to migration by coculture with M-MDSCs, compared with monocyte group. Arginase inhibitor and L-arginine abrogated the immune

suppressive function and induction of HCAECs migration of hemodialysis related M-MDSC. Plasma IFN-γ, TNF-α and IL-6 were elevated in hemodialysis patients compared with healthy control. M-MDSC level was positively related to IL-6 level among hemodialysis patients. The plasma of hemodialysis patients induced M-MDSCs significantly compared with plasma from health donors. Besides, IL-6 neutralizing antibody significantly abrogated the induction. Neutralizing antibody of IFN-γ and TNF-α partially decreased the generation of arginase of the induced M-MDSC.

Conclusions: M-MDSCs were elevated in ESRD patients under hemodialysis, and they exhibited a strong association with the risk of cardiovascular and cerebrovascular diseases. Hemodialysis related M-MDSC presented enhanced recruitment to atherosclerotic lesions, promoted the migration of endothelial cells through exhaustion of local L-arginine.

Keywords: monocytic myeloid-derived suppressor cell, hemodialysis, end stage renal disease, cardiovascular diseases, arginase

INTRODUCTION

Cardiovascular and cerebrovascular diseases are the top causes of death for end-stage renal disease (ESRD) patients and contribute to approximately half of the mortality rate (1, 2). The latent mechanism of cardiovascular and cerebrovascular diseases among ESRD patients is different from that among the general population (3). ESRD patients exhibit reverse associations with traditional risk factors of cardiovascular diseases compared to the general population (3). In contrast to the general population, obesity, hypercholesterolemia, and hypertension paradoxically appear to be protective features (3). Therefore, the specific mechanism of cardiovascular vasculopathy in the context of ESRD needs to be investigated.

Macrophages in atherosclerotic lesions are partially derived from monocytes recruited by the pathologic endothelial cell layer of blood vessels. They participate in a maladaptive, non-resolving inflammatory response that expands the subendothelial layer, which subsequently leads to necrotic cores and development of acute thrombotic vascular disease (4, 5). In our previous study, we found that HLA-DR$^{-/low}$CD11b^{+}CD14^{+}CD15^{-} cells were increased in ESRD patients after hemodialysis (6), and they could be monocytes or monocytic myeloid-derived suppressor cells (M-MDSCs) (7, 8). Monocytes and M-MDSCs were believed to transform into macrophages in multiple backgrounds, including tumor microenvironments (8, 9). Thus, we speculated that hemodialysis-related HLA-DR$^{-/low}$CD11b^{+}CD14^{+}CD15^{-} cells might contribute to the vasculopathy for ESRD patients.

In the present study, we found that hemodialysis-related HLA-DR$^{-/low}$CD11b^{+}CD14^{+}CD15^{-} cells were M-MDSCs with immune suppression capability, and were correlated with cardiovascular and cerebrovascular diseases and hazarded the overall survival (OS) of hemodialysis patients. Hemodialysis related M-MDSC presented enhanced recruitment to atherosclerotic lesions, promoted the migration of endothelial cells through exhaustion of local L-arginine, which might be associated with the mechanism of ESRD related atherosclerosis.

MATERIALS

Selection of Patients and Healthy Donors

All ESRD patients undergoing hemodialysis and healthy controls were screened for serum HIV antibody, hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV) antibody, hepatitis D virus (HDV) antigen, and HDV antibody. Patients and healthy controls who were positive for HIV tests, had chronic hepatitis virus infection, were pregnant, received systematic corticosteroids or immunosuppressive agents, presented a fever or acute infections (*e.g.*, pneumonia or urinary tract infection) within one week before recruitment, as well as those short of fundamental baseline data or reluctant to participate were excluded from this study (**Figure S1**). The study was approved by the Clinical Ethics Review Board of the Third Affiliated Hospital of Guangzhou Medical University and the Third Affiliated Hospital of Sun Yat-sen University. A written informed consent was obtained from all patients at the time of recruitment.

Baseline characteristics related to the prognosis or immunity of hemodialysis patients were collected, such as age, gender, white blood cell count, red blood cell count, neutrophil count, monocyte count, and so on, before initiation of hemodialysis and MDSC testing. For patients reluctant to pay for the above-mentioned routine tests, the tests were paid for by the funds for the present study.

Flow Cytometric Analysis

The cell phenotypes were analyzed by flow cytometry on the FACSAria II flow cytometer (BD Biosciences), and data were analyzed with the FlowJo V10.0.7 (FlowJo, Ashland, OR). For flow cytometric sorting, the BD FACSAria cell sorter (BD Biosciences) was used. The strategy for M-MDSC sorting was selection of CD11b^{+}CD14^{+}HLA-DR$^{-/low}$CD15^{-} from live peripheral blood mononuclear cells (PBMCs), with selection of CD3^{+} T cells from live PBMCs. For intracellular cytokine staining, PBMCs were stimulated in complete RPMI 1640 (Life

Technologies) with 50 ng/ml PMA (Sigma-Aldrich), 1 mg/ml ionomycin (Sigma-Aldrich), and 1 mg/ml brefeldin A (Invitrogen) for 4 h. Cells were then stained with the Abs against surface markers, fixed, and permeabilized using an Intracellular Fixation and Permeabilization Buffer Set (eBioscience). After that, cells were stained with the antibodies against cytokines. The experiments were performed in a biosafety laboratory. Agents used were listed in **Table S1**.

T Cell Proliferation and Activation Assay

T cell proliferation was determined by CFSE (5,6-carboxyfluoresceindiacetate, succinimidylester) dilution. T cells were sorted from hemodialysis patients and/or healthy donor, and used for further analysis to avoid the heterogeneity in proliferation capacity of T cells from different donors (10, 11). Purified T cells were labeled with CFSE (3 mM; Invitrogen, Carlsbad, CA), stimulated with 0.5 mg/ml of pre-coated anti-CD3 and 0.5 mg/ml anti-CD28 (eBioscience), then cultured either alone or co-cultured with M-MDSCs at the indicated ratios for three days. The cells were then stained for surface marker expression with CD4-PE or CD8-APC antibodies, and T cell proliferation was analyzed on a flow cytometer. All cultures were carried out in the presence of 20 IU/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ) in RPMI 1640 (Life Technologies, Carlsbad, CA) for three days at 37°C. Where indicated, 1 mM L-arginine, 0.5 mM nor-NOHA (an arginase I specific inhibitor; Cayman Chemicals, Ann Arbor, MI), or 100 μM L-NMMA (an iNOS inhibitor; Cayman Chemicals, Ann Arbor, MI), was added to the culture on day zero.

ELISA

Culture supernatants of the T cell and MDSC co-culture system, plasma of patients and donors were collected for ELISA testing. Interferon-γ (IFN-γ), IL-6, and tumor necrosis factor-α (TNF-α) quantification in the supernatants was determined by an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (**Table S2**).

Arginase Activity Assay

The activity of arginase was measured in cell lysates. Briefly, cells were lysed with 0.1% Triton X-100 for 30 min, then 25 mM Tris-HCl and 10 mM $MnCl_2$ were added. The enzyme was activated by heating at 56°C for 10 min. Arginine hydrolysis was performed by incubating the lysate with 0.5 M L-arginine for 120 min at 37°C. After the addition of a-isonitrosopropiophenone (dissolved in 100% ethanol), the urea concentration was measured at 540 nm, followed by heating at 95°C for 30 min.

Nitric Oxide Assay

The nitric oxide (NO) content in plasma was measured using the QuantiChrom Nitric Oxide Assay Kit, following the manufacturer's instructions (BioAssay Systems, Hayward, CA). Culture media samples (150 μl) were first mixed with ZnSO4 (8 μl) and vortexed; then NaOH (8 μl) was added, followed by centrifugation for 10 min at 14,000 rpm. 100 μl of the deproteinized supernatants was transferred to a clean tube, mixed with a combination of 100 μl of Reagent A, 4 μl of Reagent B, and 100 μl of Reagent C, and incubated for 10 min at 60°C. The absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

Quantitative Reverse Transcription Polymerase Chain Reaction

RNA was extracted with the Multisource Total RNA Miniprep Kit (Axygen, Union City, CA), and quantitative reverse transcription polymerase chain reaction was performed utilizing commercially available primers (**Table S3**) and SYBR Premix ExTaq II (Code, DRR081; Takara Biotechnology Co., Dalian, China). Fluorescence for each cycle was quantitatively analyzed using the ABI Prism 7000 sequence detection system (Life Technologies). The results were reported as relative expression, normalized with the GAPDH housekeeping gene as an endogenous control. Monocytes from one health donor were used as control and compared by the monocytes from other health donors and M-MDSC from hemodialysis patients. The relative mRNA expression was displayed in arbitrary units.

Migration Assays

Human coronary arterial endothelial cells (HCAECs) (Lonza, Basel, Switzerland) were cultured in EGM-2 media (Lonza). Cell migration was assessed using a 24-well QCM Chemotaxis cell migration assay (Millipore) with 8 mm pore size as per the manufacturer's instructions. Cells were resuspended in EBM-2 serum-free medium, counted and plated onto the up chamber at 37,500 cells per chamber in triplicate. Cells were allowed to migrate overnight towards EBM-2 media + 10% FBS at 37°C. Monocytes from health donors, M-MDSCs from hemodialysis patients were added at a 1:10 ratio (M-MDSCs: HCAECs), with 1 mM L-arginine or 0.5 mM nor-NOHA administrated as indicated. The cells in the lower chambers were then fixed with 4% paraformaldehyde for 10 min, washed once with PBS solution, stained with crystal violet for 10 min, washed again with PBS solution, and finally counted and photographed with a Leica DC300F digital microscope (Leica Camera AG, Solms, Germany).

In Vitro Monocytic Myeloid-Derived Suppressor Cells Generation From Peripheral Blood Mononuclear Cell

PBMCs from health donor cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 20 ng/ml GM-CSF, and 50 μM 2-ME. The cultures were maintained at 37°C in 5% CO_2-humidified atmosphere in 24-well plates. Medium was refreshed on day 3. Cells were analyzed by flow cytometry on day 5. 20 ng/ml of IL-6 neutralizing mAb (IL-6 mAb, R&D Systems), 10 μg/ml of infliximab (MSD, Whitehouse Station, NJ) or 1 μg/ml of both anti-IFN-γ (R&D Systems, MAB285, clone # 25718) and anti-IFN-γR1 (R&D Systems, MAB6731, clone # 92101) was added with PBS as vehicle control.

Patient Follow-Up and Statistical Analysis

Patients returned for follow-up appointments at least every week until death. The follow-up duration was calculated from the first day of M-MDSC testing to the day of death, or to the last follow-up. OS was the end point, which was calculated from the first day of treatment to death (12).

Variables in different groups were compared using the χ^2 test (or the Fisher's exact test, if indicated) and t-test or the non-parametric Mann–Whitney U tests. OS was calculated using the Kaplan–Meier survivor function and with Kaplan–Meier failure function conducted to illustrate occurrence of stroke events, acute myocardial infarction (AMI) events, and heart failure events since the day of M-MDSC testing. Their differences were compared using the log-rank test. Multivariate analysis using a Cox proportional hazards model was used to test for independent significance by entry of insignificant explanatory variables. Covariates including host factors (*i.e.*, age and gender), characteristics of MDSC testing, and MDSC subsets were included in all tests. For *in vitro* experiments, statistical analyses were done using paired t-tests. Correlations between different parameters were analyzed using the Spearman's rank test (13). The criterion for statistical significance was set at $\alpha = 0.05$ and all *P*-values were based on two-sided tests. Statistical tests were performed using GraphPad Prism version 5.0a and SPSS Statistics 20.0. P values of 0.05 were considered significant.

RESULTS

Monocytic Myeloid-Derived Suppressor Cells Were Elevated in Hemodialysis Patients and Decreased Overall Survival

During the period between October 2015 and December 2015, we investigated a series of 104 ESRD patients undergoing maintenance hemodialysis in the Third Affiliated Hospital of Guangzhou Medical University and the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. The ESRD was considered irreversible by two independent nephrologists rather than representing acute kidney injury. Age- and gender-matched healthy controls (n = 60) consisted of local volunteers. The complications of the 104 hemodialysis patients when testing M-MDSC were documented, which revealed that 36 patients presented diabetes, 31 presented stable coronary heart disease, 72 patients presented chronic heart failure, and 30 patients presented cerebrovascular disease. A total of 37 patients died before the ending of this study. The major death causes were heart diseases and stroke (**Table S4**). The complications of the health donor were all stable, with none of them presented poorly controlled diseases such as diabetes, hypertension, cardiovascular disease, and cerebrovascular disease (**Table S5**).

Blood samples were collected within 1 h before daily hemodialysis during maintenance hemodialysis. PBMCs were isolated from whole blood by Ficoll centrifugation and analyzed within 6 h of blood sampling. Circulating frequencies of the subsets of MDSCs were quantified with the gating strategy indicated. M-MDSCs were HLA-$DR^{-/low}CD11b^{+}CD14^{+}CD15^{-}$ and polymorphonuclear-MDSCs (PMN-MDSCs) were HLA-$DR^{-/low}CD11b^{+}CD14^{-}CD15^{+}$ (**Figure 1A**). Patients with ESRD undergoing hemodialysis presented slightly increased PMN-MDSCs (**Figure 1B**) and a significantly higher level of M-MDSCs (**Figure 1C**) compared to that in healthy controls.

Multivariate Cox analysis revealed that M-MDSC and age were the independent prognostic factors for OS among hemodialysis patients (**Table S6**). Besides, PMN-MDSC levels were dichotomized to high and low level according to the median value. Kaplan–Meier analysis revealed that they were not associated with OS. (**Figure 1D**). Thus, we focused on M-MDSC in further analysis.

M-MDSC were tested 3 months after first testing among 103 hemodialysis patients, with one patient not retested due to early death. The repeated results of M-MDSC levels were consistent with the initial results (**Figure 1E**). M-MDSC levels were dichotomized to high and low levels according to the median value of each testing. Among the 103 patients, 21 changed their category of M-MDSC levels (Intermediate group). M-MDSC levels of 10 patients changed from high level in the initial test to low level in the second test, with the rest 11 patients changed reversely. 41 patients displayed persistent low M-MDSC levels, with the rest 42 patients presenting persistent high M-MDSC levels (**Figure S2**). More patients died at the end of this study among the patients with high M-MDSC levels (p = 0.035) (**Table S4**). Patients with persistent high M-MDSC levels presented higher lymphocyte and monocyte counts and lower serum creatine when testing M-MDSC. They displayed better eGFR before initiation of hemodialysis (**Table S4**). Survival analysis revealed that patients with persistent high level of M-MDSC presented the worst OS (**Figure 1F**).

Monocytic Myeloid-Derived Suppressor Cells Contributed to Hemodialysis-Related Cardiovascular Diseases

Patients with high levels of M-MDSCs presented decreased OS, but the reasons had to be identified. In our previous study we found that M-MDSCs did not influence the risk of infectious disease (6), which was the second leading cause of mortality for ESRD patients. Then, we analyzed the association of M-MDSCs with cardiovascular and cerebrovascular diseases, which were the major causes of mortality for ESRD patients (1, 2). Stroke, AMI, and acute heart failure events were documented. Kaplan–Meier failure analysis displayed that patients with persistent high M-MDSC levels presented significantly higher risk of stroke and heart failure than patients with low and intermediate M-MDSC levels. But M-MDSC levels did not influence AMI events (**Figure 2A**). Correlation analysis revealed that M-MDSC levels were positively related to blood urine nitrogen (BUN), monocyte count, and creatinine (**Figure 2B** and **Figure S3**). Hemodialysis patients with coronary heart disease and cerebrovascular disease presented significantly higher M-MDSC levels than those without the complications. Diabetes and chronic heart failure did not influence the M-MDSC levels (**Figure 2B**). Multivariate Cox regression revealed that M-MDSC level was an independent

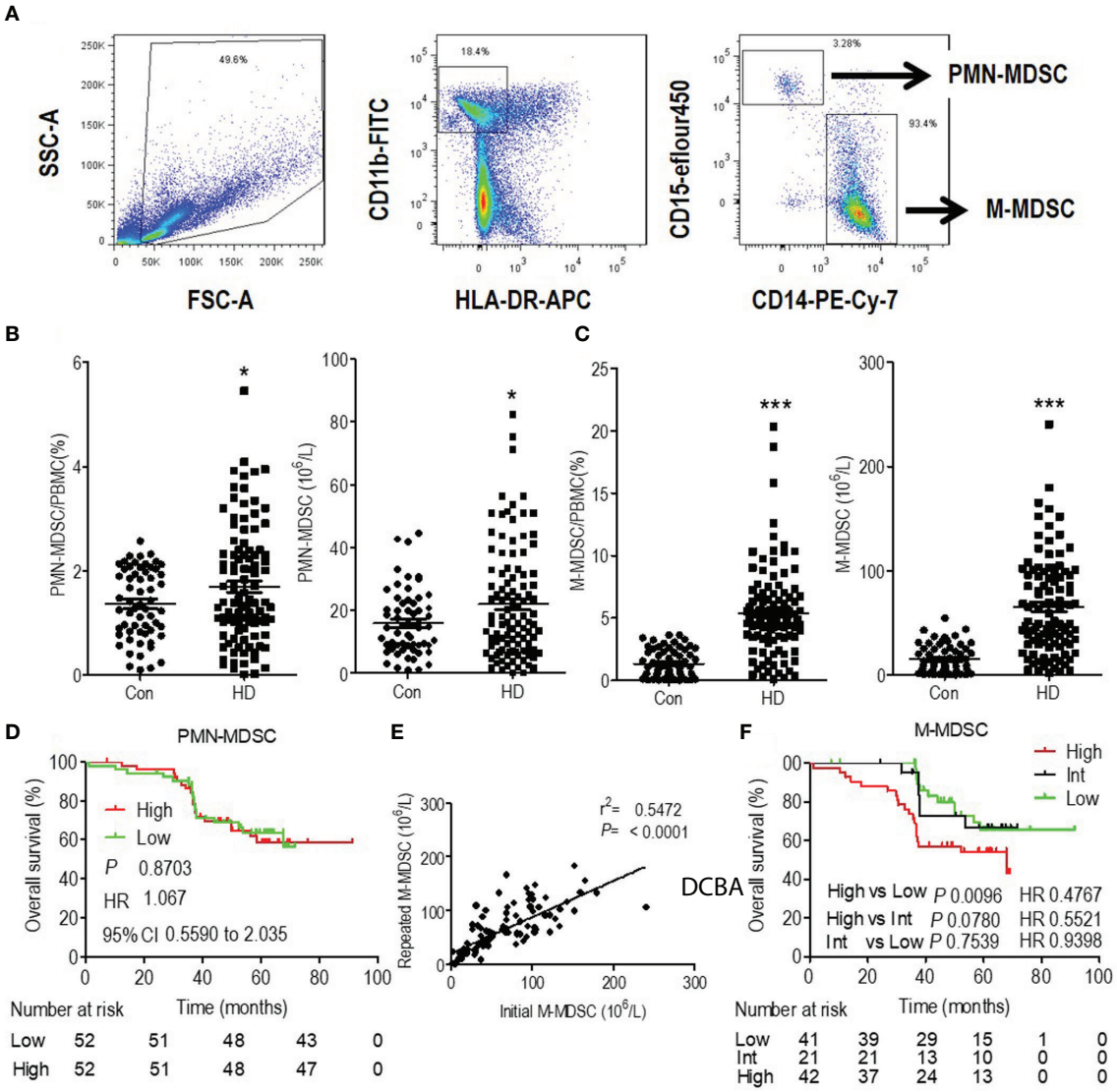

FIGURE 1 | Expansion and prognostic value of monocytic myeloid-derived suppressor cells (M-MDSCs) in end-stage renal disease (ESRD) patients undergoing hemodialysis. **(A)** Gating strategy of monocytic MDSCs (M-MDSCs) by flow cytometry analysis. Polymorphonuclear-MDSC (PMN-MDSC) was defined as $CD11b^{+}CD15^{+}CD14^{-}HLA\text{-}DR^{-/low}$, with M-MDSC defined as $CD11b^{+}CD15^{-}CD14^{+}HLA\text{-}DR^{-/low}$. Frequency and absolute count of PMN-MDSCs **(B)** and M-MDSCs **(C)** in the peripheral blood of healthy controls and ESRD patients under hemodialysis (HD). *P < 0.05, ***P < 0.001. **(D)** Kaplan–Meier survival curves are shown for overall survival in low and high levels of PMN-MDSC hemodialysis patients. **(E)** Linear regression analysis of the association between initial M-MDSC results and repeated M-MDSC results (3 months later). **(F)** Kaplan–Meier survival curves are shown for overall survival in low, intermediate (Int) and high levels of M-MDSC hemodialysis patients. For Kaplan–Meier survival curves, hazards ratios (HRs) were calculated using the unadjusted Cox proportional hazards model. *P* values were calculated using the unadjusted log-rank test. 95% CI indicates 95% confidence interval.

prognostic factor for stroke event, but not for heart failure and AMI (**Tables S7–S9**). Concerning other prognostic factors, age was related to AMI events (**Table S7**). Lower serum creatinine, higher serum albumin, and urea reduction rates predicted heart failure events (**Table S8**). Age, white blood cell counts, neutrophils counts, monocyte counts predicted stroke events (**Table S9**). Above all, M-MDSCs were confirmed to be a prognostic factor for cardiovascular and cerebrovascular diseases among hemodialysis patients, especially for stroke events.

Monocytic Myeloid-Derived Suppressor Cells in Hemodialysis Patients Presented Immune Suppressive Function

Since M-MDSCs share the same surface marker with monocytes, $HLA\text{-}DR^{-/low}CD11b^{+}CD14^{+}CD15^{-}$ cells in the present study might be M-MDSCs or monocytes (7). Thus, it was imperative to define whether these cells were M-MDSCs or monocytes. M-MDSCs are characterized by their suppressive capability on T cell response because they share the same definitive markers as

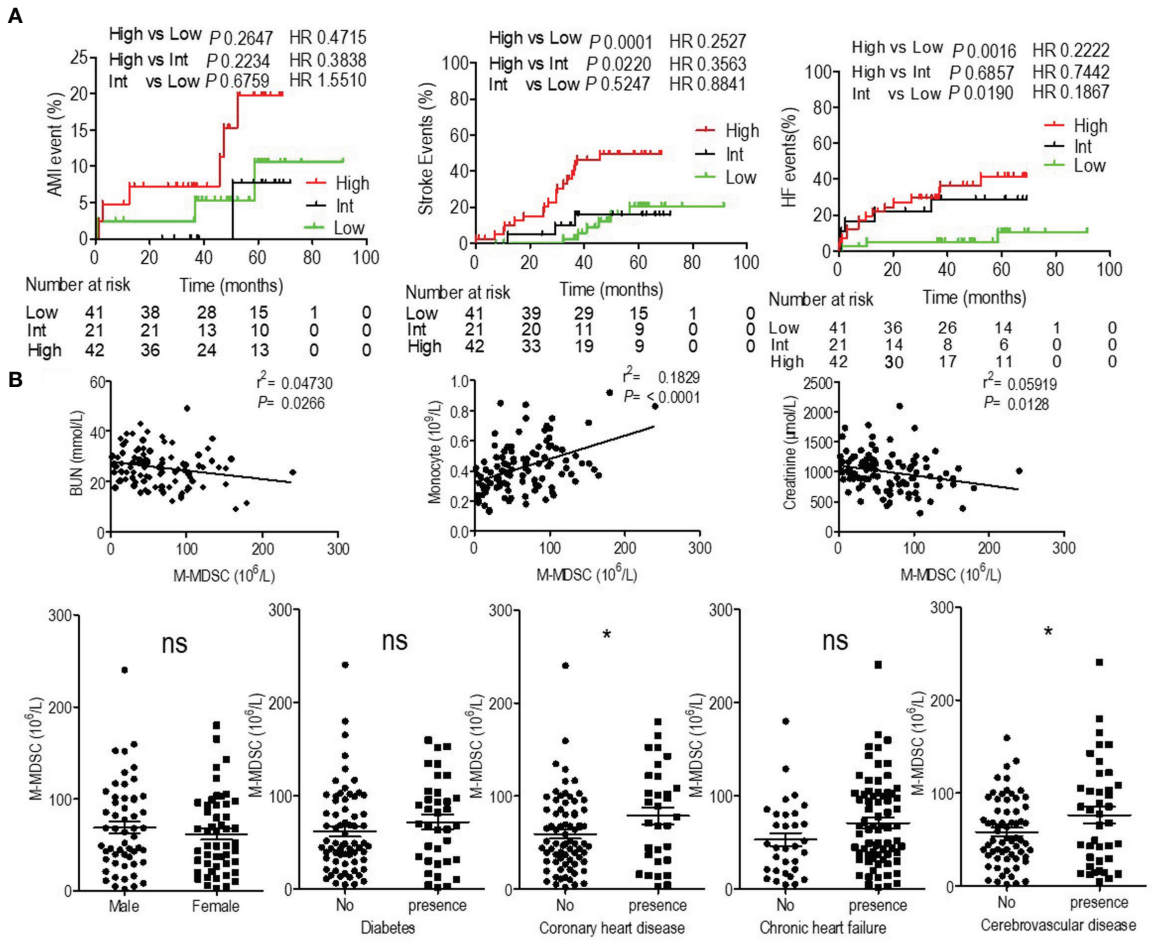

FIGURE 2 | Prognostic value of monocytic myeloid-derived suppressor cells (M-MDSCs) for cardiovascular diseases in end-stage renal disease (ESRD) patients under hemodialysis and their correlation with clinical characteristics. **(A)** Kaplan–Meier survival curves are shown for acute myocardial infarction (AMI), heart failure (HF), and stroke-free survival in low, intermediate (Int) and high levels of M-MDSCs hemodialysis patients. Hazard ratios (HRs) were calculated using the unadjusted Cox proportional hazards model. *P* values were calculated using the unadjusted log-rank test. **(B)** Correlation with clinical characteristics.

their normal counterparts, monocytes (6–8, 14). To investigate whether M-MDSCs in ESRD patients undergoing hemodialysis suppressed T cell response, T cells and M-MDSCs were purified from PBMC using flow sorting. CFSE-labeled PBMC-derived CD3 T cells were stimulated with anti-CD3 and anti-CD28, with the indicated ratio of M-MDSCs in a co-culture system. CD4 and CD8 T cell proliferations were significantly abrogated by the addition of hemodialysis-related M-MDSCs in a dose-dependent manner. The IFN-γ levels in the media, tested using ELISA, illustrated that IFN-γ secretion was decreased after administration of hemodialysis-related M-MDSCs. Meanwhile, monocytes from healthy donors did not exhibit a suppressive function. (**Figures 3** and **S4A, B**) Then, cross-validation experiments with co-cultures were conducted to verify the results. Monocytes did not suppress the proliferation and activation of T cells from both health donors and hemodialysis patients. M-MDSC from hemodialysis suppressed T cells from both health donors and hemodialysis patients. (**Figures S5A–D**) T cells from hemodialysis patients did not proliferate after stimulation as well as those from the health donors (**Figures S5E, F**). M-MDSCs existed in ESRD patients with hemodialysis, but not in healthy donors. Above all, HLA-DR$^{-/low}$ CD11b^{+}CD14^{+}CD15^{-} cells in hemodialysis patients were M-MDSCs.

Monocytic Myeloid-Derived Suppressor Cells Presented Higher Cell Surface Adhesion Molecules

Recruitment of monocytes to the vessel wall was reported to be an early step in the formation of atherosclerotic lesions (4, 15). Recruited monocytes induced local inflammation and were a source of foam cells (5). Thus, to investigate the recruitment behavior of M-MDSCs, the expression of adhesion molecules was investigated using qRT-PCR. As a result, M-MDSCs presented higher levels of CXCR4 and VLA-4 compared to those of monocytes, with similar levels of PSGL1, L-selectin,

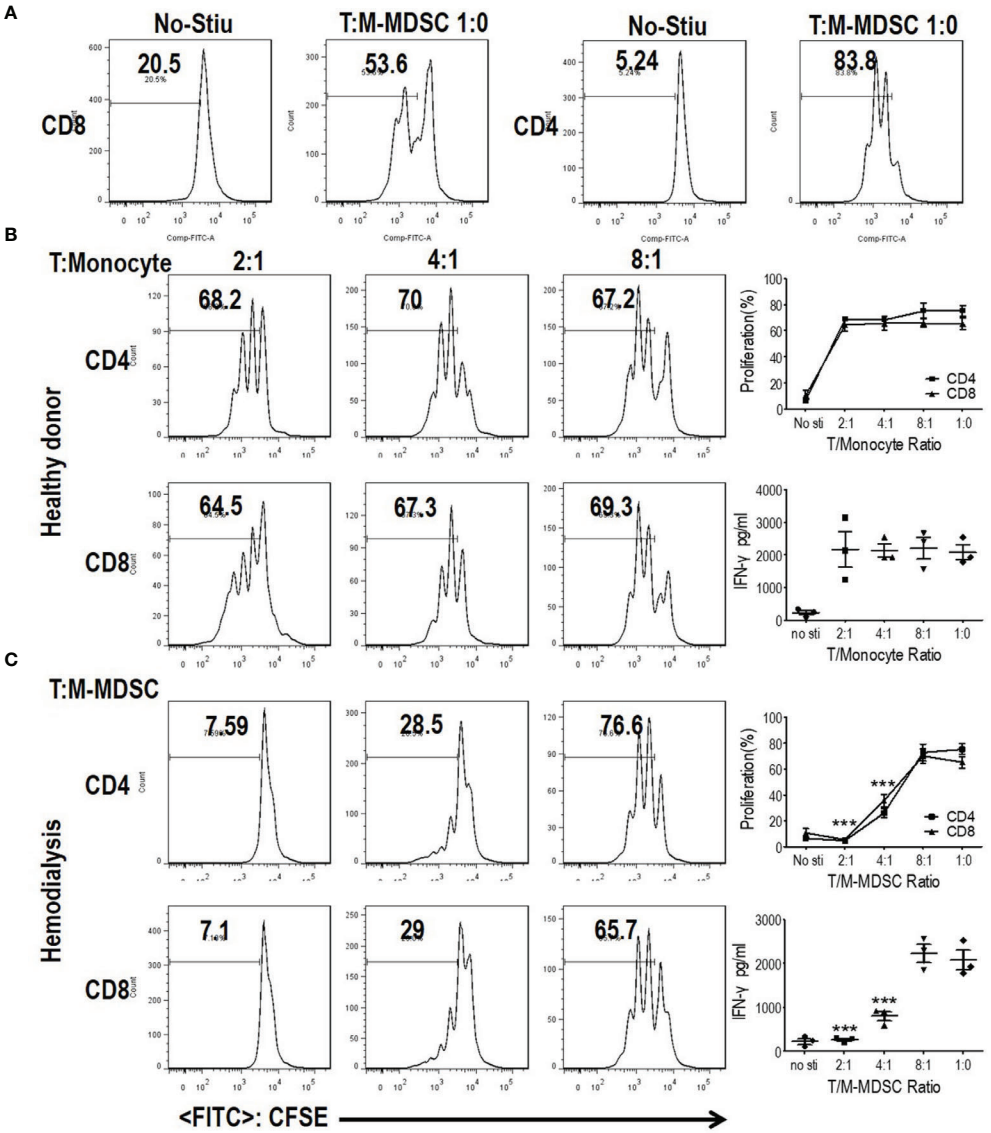

FIGURE 3 | Monocytic myeloid-derived suppressor cells (M-MDSCs) from end-stage renal disease (ESRD) patients under hemodialysis suppressed T cell proliferation and activation. $CD3^+$T cells from peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD3 and anti-CD28, co-cultured with M-MDSCs from the same donors at different ratios for 3 days, and evaluated for $CD4^+$ and $CD8^+$ T cell proliferation by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and interferon-γ production in supernatants by enzyme-linked immunosorbent assay. **(A)** Representative flow cytometry data of positive control and negative control. **(B)** Results of healthy controls. **(C)** Results of patients with ESRD. Left panels: Representative flow cytometry data from one individual. Right panels: Cumulative data (n = 3) and concentration of interferon-γ in the media (n = 3). ***P < 0.001.

P-selectin, and E-selectin. Thus, compared to monocytes, hemodialysis-related M-MDSCs were more likely to be recruited to atherosclerotic lesions (**Figure 4**).

Elevation of S100A9 was reported to be an important upstream molecular event for the development of M-MDSCs (7, 16, 17). S100A9 was tested in hemodialysis-related M-MDSCs and monocytes, thus revealing that S100A9 had significantly higher expression in M-MDSCs (**Figure 4**). This result indicated that S100A9 might induce the expression of CXCR4 and VLA-4.

Monocytic Myeloid-Derived Suppressor Cells Induced Migration of Vascular Endothelial Cell by Arginine Deprivation

Recruited monocytes played vital roles in both early atherogenesis and advanced plaque progression by multiple mechanisms including transformation into macrophages (4, 5, 18). Thus, to investigate the mechanism of the M-MDSC-induced acceleration of cardiovascular disease, we tested the bioactive substances which were reported to be generated by M-MDSCs from a tumor background. Based on the finding that suppression of T cell

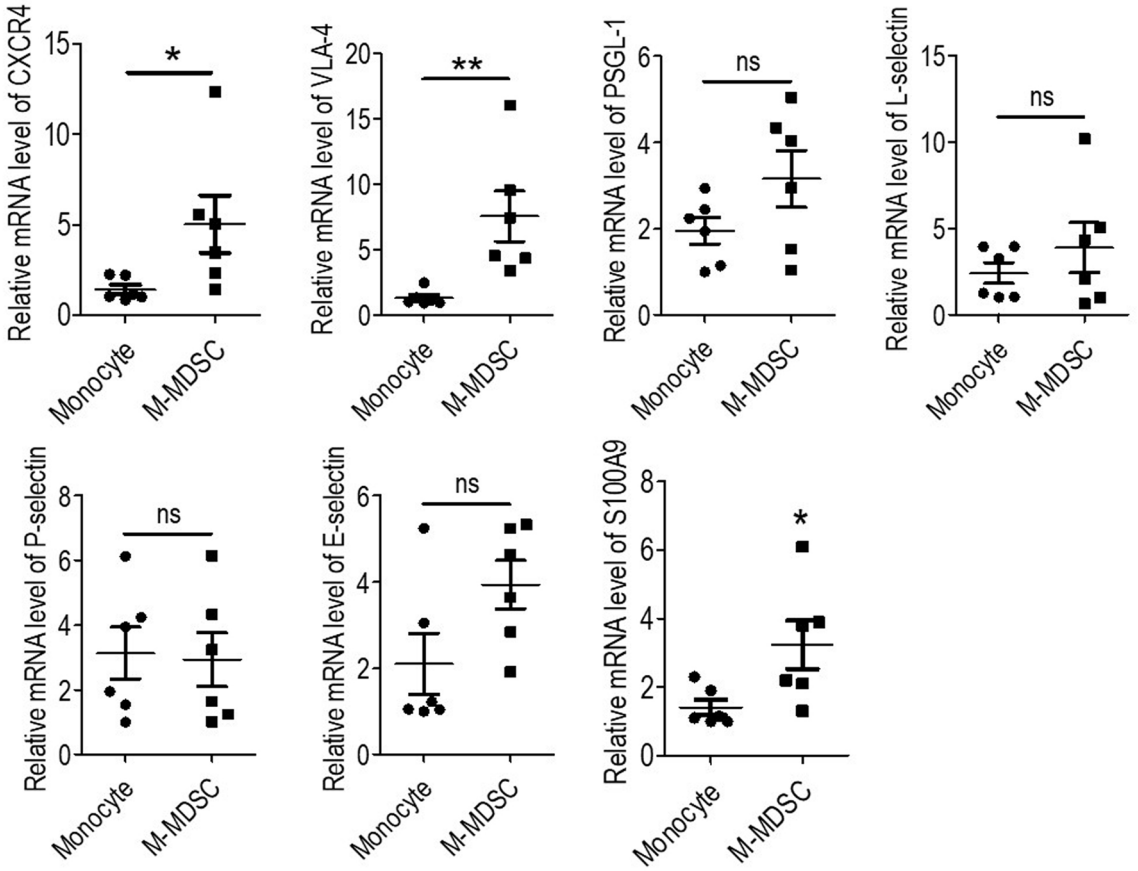

FIGURE 4 | Expression of cell surface adhesion molecules and S100A9 of monocytic myeloid-derived suppressor cells (M-MDSCs). Expression of CXCR4, VLA-4, PSGL-1, L-selectin, P-selectin, E-selectin, and S100A9 were evaluated by qRT-PCR in M-MDSCs and monocytes. (n = 6). *P < 0.05; **P < 0.01.

proliferation was a feature of M-MDSCs differing from monocytes, we further explored the underlying mechanisms controlling hemodialysis-related M-MDSC-mediated T cell suppression. Previous reports confirmed that arginase I or NO were immune mediators for M-MDSC-mediated immune suppression (7, 8, 14, 19). Thus, in M-MDSCs and T cell proliferation (ratio 1:2) co-culture system, an arginase inhibitor (N ω-hydroxy-nor-l-arginine, nor-NOHA), L-arginine supplementation, or an inducible nitric oxide synthase (iNOS) inhibitor (N^G-Monomethyl-L-arginine, L-NMMA) was utilized to reverse the suppressive effects of M-MDSCs on T cell proliferation in a co-culture system (20). Nor-NOHA, L-arginine and L-NMMA did not influence the proliferation of T cells (**Figure S6**). As a result, the suppression on T cell proliferation and IFN-γ production was reversed by arginase inhibitor, nor-HOHA, and L-arginine (**Figures 5A** and **S4C**). Meanwhile, the expression of arginase I and activity of arginase were also significantly raised in hemodialysis-related M-MDSCs compared to monocytes from healthy donors (**Figures 5B, C**).

Decreased L-arginine was reported as critically important for endothelial nitric oxide synthase (eNOS) uncoupling, which led to decreased NO production, increased ROS generation, and finally the development of atherosclerosis (21, 22). Migration of vascular endothelial cells is a critical process for the development of atherosclerosis (23). In order to prove the M-MDSCs were involved in the development of atherosclerosis, M-MDSCs from hemodialysis patients were co-cultured with HCAECs. HCAECs presented increased capability to migration in M-MDSC group compared with monocyte group. Arginase inhibitor, nor-HOHA, and L-arginine reversed this phenomenon (**Figures 5D, E**). Thus, M-MDSCs might promote the migration of endothelial cells into vascular intima through deprivation of L-arginine.

Monocytic Myeloid-Derived Suppressor Cells Might be Induced by Elevation of Interferon-γ, Tumor Necrosis Factor-α, and IL-6 in Hemodialysis Patients

According to previous reports, IFN-γ and TNF-α activated the immune suppressive function of M-MDSCs, and IL-6 induced M-MDSCs' expansion (24). Thus, we test IFN-γ, TNF-α, and IL-6 in the plasma of hemodialysis patients collected when testing M-MDSCs. We found that plasma IFN-γ, TNF-α and IL-6 was elevated in hemodialysis patients compared with

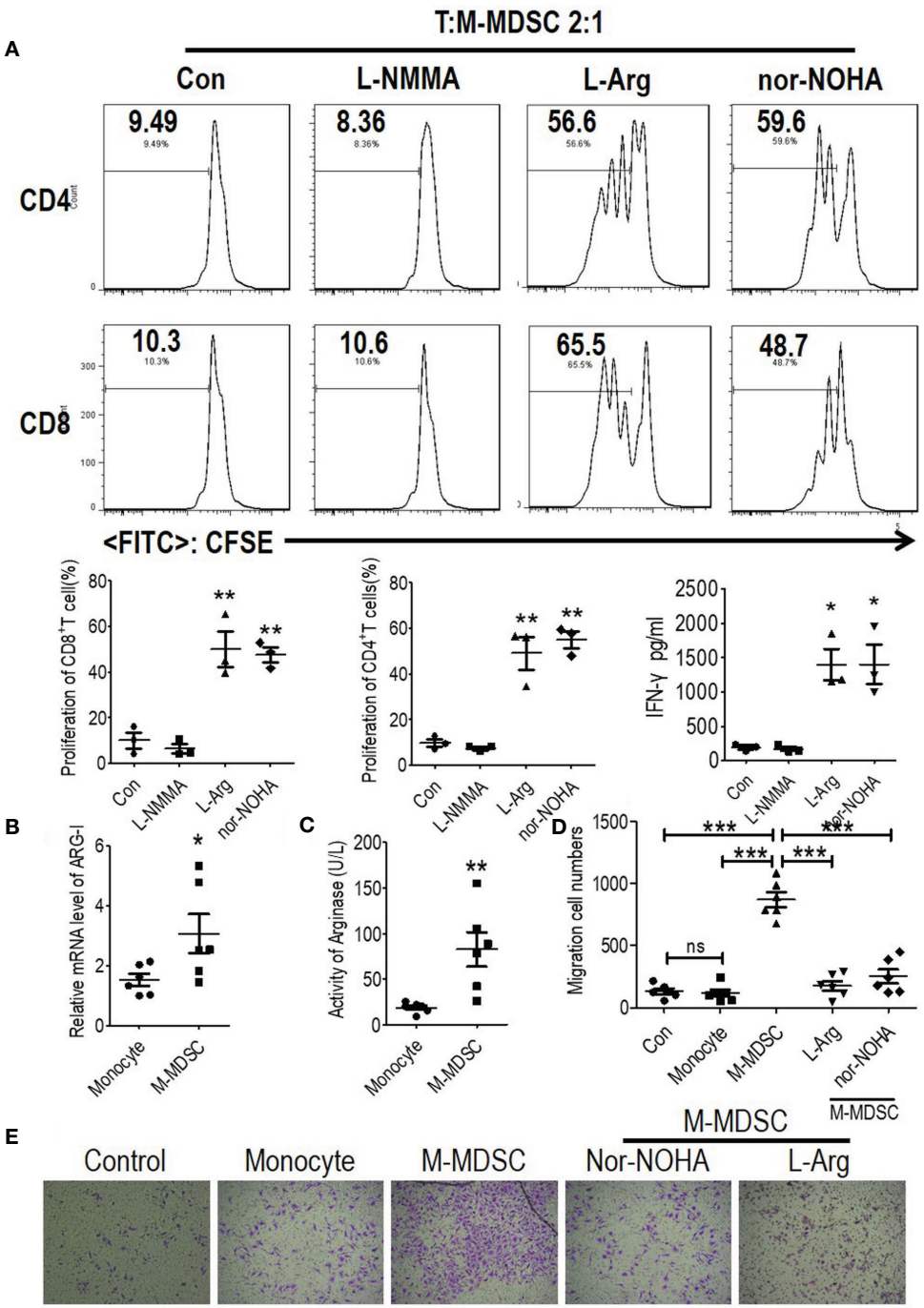

FIGURE 5 | Monocytic myeloid-derived suppressor cells (M-MDSCs) suppressed functional T cells and promoted human coronary arterial endothelial cells (HCAECs) migration in an arginase 1-dependent manner. **(A)** Effect of arginase inhibitor NOHA, L-arginine supplementation, or inducible nitric oxide synthase (iNOS) inhibitor L-NMMA on M-MDSC function. T cells from ESRD patients were stimulated with anti-CD3/anti-CD28, cocultured with M-MDSCs from whole blood at a 2:1 ratio with treatments as indicated, evaluated for T cell proliferation by CFSE labeling, and IFN-γ production in supernatants by ELISA. Representative flow cytometry data, cumulative data (n = 3), and concentration of IFN-γ in the media (n = 3). Expression of Arg1 **(B)** and arginase activity **(C)** in M-MDSCs and monocytes. (n = 6). **(D, E)** The migration of HCAECs was tested using transwell experiment with monocytes from healthy donors, M-MDSCs from hemodialysis patients added at a 1:10 ratio (M-MDSCs: HCAECs). 1 mM L-arginine or 0.5 mM nor-NOHA was added as indicated in M-MDSCs' treatment group. Cumulative data **(D)** and typical results were shown **(E)**. *P < 0.05; **P < 0.01; ***P < 0.001.

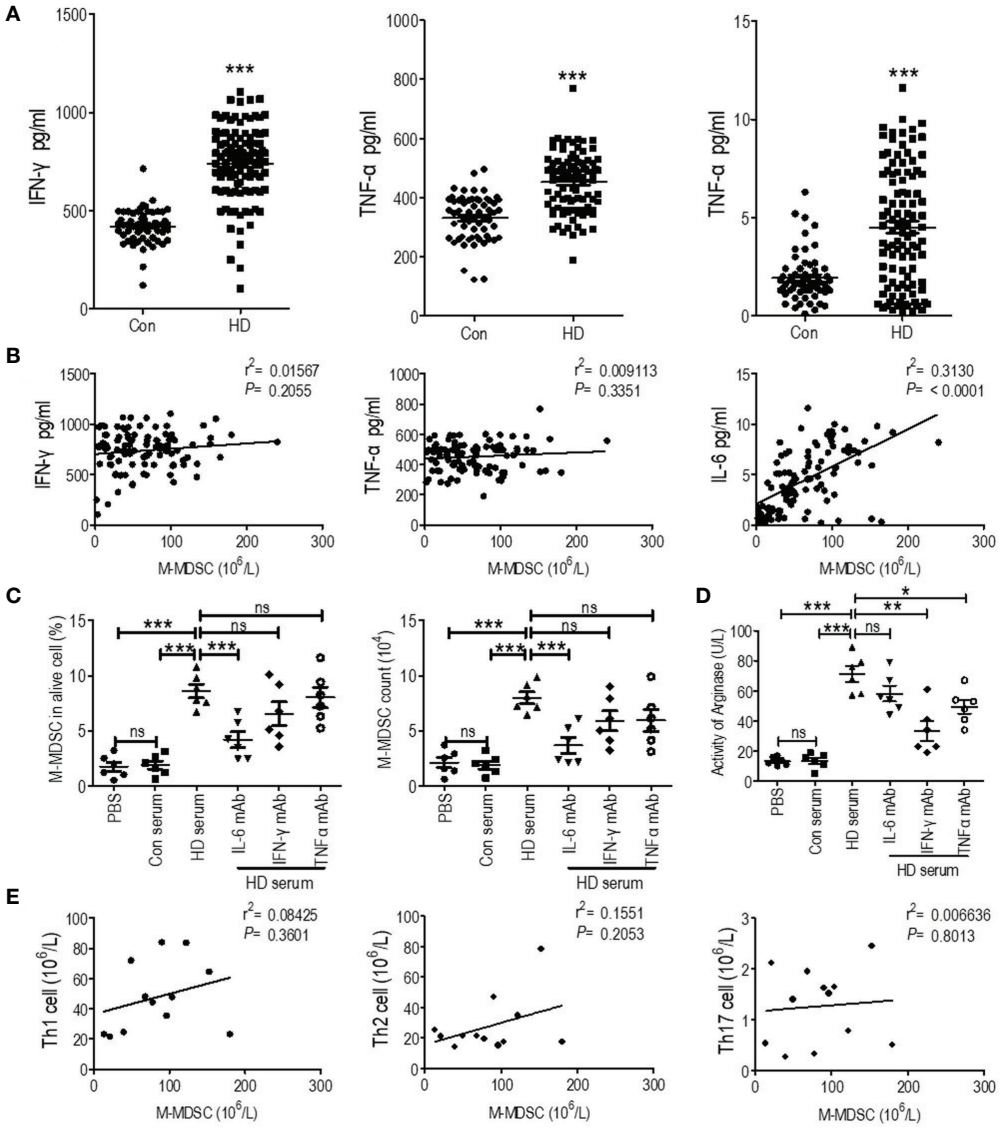

FIGURE 6 | Correlation of plasma cytokines and T helper (Th) cells with M-MDSCs among end-stage renal disease (ESRD) patients under hemodialysis. **(A)** IFN-γ, TNF-α and IL-6 levels of the plasma collected when testing M-MDSC of health donor (Con) and hemodialysis patients (HD). **(B)** Linear regression analysis of the association between M-MDSC level and IFN-γ, TNF-α, and IL-6 levels. **(C, D)** Plasma of hemodialysis patients was utilized to induce M-MDSC from PBMC of healthy donor with or without neutralizing antibody of IL-6, IFN-γ, and TNF-α. The percentage in alive cells and absolute cell counts of M-MDSC **(C)** and the activity of arginase of induced M-MDSCs **(D)** in each group were shown (n = 6). **(E)** Linear regression analysis of the association between M-MDSC results and Th1, Th1, and Th17 levels (n = 12). *P < 0.05; **P < 0.01; ***P < 0.001.

healthy controls (**Figure 6A**). Notably, linear regression found positive association between M-MDSC levels and IL-6 levels among hemodialysis patients (**Figure 6B**). IL-6 predicted OS, and IFN-γpredicted AMI events and stroke events. Then, plasma of hemodialysis patients and that from health donors was utilized to induce M-MDSCs from PBMCs of healthy donors. As a result, the plasma of hemodialysis patients induced M-MDSCs significantly compared with the plasma from healthy donors. Besides IL-6 neutralizing antibody partially abrogated the induction (**Figure 6C**). Neutralizing antibodies of IFN-γ and TNF-α partially decreased the generation of arginase of the induced M-MDSCs (**Figure 6D**). Thus, our results indicated that the elevation of plasma IL-6 in the hemodialysis patients might be one of the causes of M-MDSCs' elevation, with the increase of IFN-γand TNF-α associated with activation of M-MDSCs.

Besides, Th1, Th2, and Th17 cells were tested in 12 hemodialysis patients and 12 healthy control (**Figure S6A**). They were all elevated in hemodialysis patients (**Figure S7**). However, their levels were not associated with M-MDSC levels (**Figure 6E**).

DISCUSSION

Cardiovascular and cerebrovascular diseases are the leading cause of mortality for ESRD patients (1, 2). However, the mechanism of ESRD-related cardiovascular diseases for these patients was considered quite different from that of the general population. Interestingly, ESRD patients exhibit reverse associations with traditional risk factors for cardiovascular diseases. Hemodialysis was considered a critical cause of cardiovascular and cerebrovascular diseases due to severe hemodynamic changes (2). Obesity, hypercholesterolemia, and hypertension paradoxically appear to be protective features, opposite to the general population (3). Thus, the novel mechanism of ESRD-related cardiovascular diseases was investigated. In our present study, we found that M-MDSCs were elevated in hemodialysis patients. And persistent high M-MDSC levels predicted OS and cardiovascular and cerebrovascular disease events. Hemodialysis related M-MDSC presented enhanced recruitment to atherosclerotic lesions and exhaustion of local L-arginine, which might be involved in the mechanism.

Monocytes and M-MDSC share the same surface marker and morphology (7, 8, 25). Monocytes play a critical role in the development of cardiovascular diseases. They could migrate to atherosclerotic lesions, transform to foam cells, and induce local metabolic changes (4, 5). However, the predictive value of circulating monocyte count for cardiovascular diseases remains under debate (26–29). Some reports suggest that circulating monocyte count predicts cardiovascular diseases in ESRD patients (26–29). In our study, monocyte count was a potential predictor of stroke events among ESRD patients. However, blood cell count could not distinguish M-MDSCs from monocytes due to their similar morphology. Thus, it was necessary to identify the nature of these cells. This present study revealed they were M-MDSCs and presented independent prognostic value for OS and stroke events among hemodialysis patients.

Hemodialysis-related M-MDSCs were confirmed to suppress T cell proliferation. However, we could not conclude that M-MDSCs promoted cardiovascular and cerebrovascular diseases by induction of immune suppression. Immune suppression capacity illustrated their nature as M-MDSCs. However, current data did not support the immune suppressive function of M-MDSC induced cardiovascular and cerebrovascular events among hemodialysis patients. Previous studies on T cells of ESRD patients revealed presence of preactivated T cells in hemodialyzed patients (30). Consequently, T cell proliferation and activation in response to mitogen are impaired (31). Our results were consistent with previous studies. We found Th1, Th2, and Th17 cells were more frequent in hemodialysis patients compared with healthy donors. And T cell proliferation and activation in response to antigen non-specific stimulation were weaker in hemodialysis patients. According to previous study, M-MDSCs exacted biological function in microenvironment instead of circulation (8). M-MDSCs in hemodialysis patients could not suppress T cells in circulation, indicated by its irrelevance with Th1, Th2, and Th17 cells and the risk of infectious diseases in our series studies (6). On the contrary, proinflammatory factors in the circulation of hemodialysis patients (32) induced M-MDSC accumulation and activation (24). Thus, hemodialysis related M-MDSCs shall not promote cardiovascular and cerebrovascular diseases by induction of immune suppression.

Hemodialysis-related M-MDSCs might promote atherosclerosis in the microenvironment of endothelial cells. We found that hemodialysis-related M-MDSCs displayed higher levels of cell surface adhesion molecules, which increased their capability to invade atherosclerotic lesions. M-MDSCs were reported to transform into macrophages in tumor microenvironment (9), which led to the speculation that M-MDSCs might be the major source of macrophages in atherosclerotic lesions. Additionally, hemodialysis related M-MDSCs were similar with the plasma of hemodialysis patients induced M-MDSC in the activity of arginase compared with their control, monocytes of health donor and plasma of health donors induced M-MDSC. Their exhaustion of local L-arginine by increased activity of arginase might be of great importance for the development of atherosclerosis lesions (21, 22). Moreover, we found migration of vascular endothelial cells, as a critical process for the development of atherosclerosis (23), was promoted by hemodialysis-related M-MDSCs through deprivation of L-arginine. Above all, hemodialysis related M-MDSC presented enhanced recruitment to atherosclerotic lesions, promoted the migration of endothelial cells through exhaustion of local L-arginine, which might be the mechanism of ESRD related atherosclerosis.

The latent causes for the induction of hemodialysis-related M-MDSCs were not clear. We found that M-MDSCs were elevated after hemodialysis (6). Plasma IFN-γ, TNF-α, and IL-6 were elevated in hemodialysis patients compared with healthy control. IL-6 was associated with the prognosis, which was similar with previous studies (33). Notably, linear regression found positive association between M-MDSC levels and IL-6 levels among hemodialysis patients. According to previous reports, IFN-γ and TNF-α induced the immune suppressive function of M-MDSC, with IL-6 causing M-MDSC expansion (24). The present study found that plasma of hemodialysis patients induced M-MDSCs significantly compared with plasma from health donors. Besides IL-6 neutralizing antibody partially abrogated the induction. Neutralizing antibody of IFN-γ and TNF-α partially decreased the generation of arginase of the induced M-MDSCs. Thus, our results indicated that the elevation of plasma IL-6 in the hemodialysis patients might be one of the causes of M-MDSC elevation, with the increase of IFN-γ and TNF-α associated with activation of M-MDSC.

Decreasing M-MDSC might contribute to less hemodialysis-related cardiovascular disease and stroke. Elimination of M-MDSC by specific antibodies was not practical due to lack of specific surface markers for human M-MDSCs. M-MDSC specific surface markers are needed to be identified in future study. A series of clinically available agents targeting MDSCs, such as all-transretinoic acid and cyclooxygenase 2 (COX2) inhibitors (8), might benefit patients with hemodialysis by suppressing MDSCs. However, the mechanisms of M-MDSC expansion and activation were diseases specific. It is necessary to investigate the mechanism of hemodialysis-related M-MDSCs. Besides, it is interesting to access the M-MDSC levels among hemodialysis patients taking all-transretinoic acid or COX2 inhibitors.

In summary, the present study found that M-MDSCs were elevated in ESRD patients under hemodialysis, and they exhibited a strong association with the risk of cardiovascular and cerebrovascular diseases. Hemodialysis related M-MDSC presented enhanced recruitment to atherosclerotic lesions, promoted the migration of endothelial cells through exhaustion of local L-arginine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Clinical Ethics Review Board of the Third Affiliated Hospital of Guangzhou Medical University and the Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL and Y-FX designed and funded this study. Y-FX, J-RC, W-YZ, and C-ML conducted sample collection and quality control as well as in all experiments. Y-FX and C-ML followed up the patients. J-RC did statistical analysis. XL and Y-FX wrote this paper and all coauthors revised it. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.577253/full#supplementary-material

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MINI REVIEW
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Current Developments of Clinical Sequencing and the Clinical Utility of Polygenic Risk Scores in Inflammatory Diseases

Matthias Hübenthal[1†], Britt-Sabina Löscher[2†], Jeanette Erdmann[3], Andre Franke[2], Damian Gola[4], Inke R. König[4] and Hila Emmert[1]*

[1] Department of Dermatology, Quincke Research Center, University Hospital Schleswig-Holstein, Kiel, Germany, [2] Institute of Clinical Molecular Biology, Christian-Albrechts University of Kiel and University Hospital Schleswig-Holstein, Kiel, Germany, [3] Institute for Cardiogenetics, University of Lübeck, Lübeck, Germany, [4] Institute of Medical Biometry and Statistics, University of Lübeck, Lübeck, Germany

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Oliver Distler,
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****Correspondence:***
Hila Emmert
hemmert@dermatology.uni-kiel.de

[†]These authors have contributed equally to this work

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In this mini-review, we highlight selected research by the Deutsche Forschungsgemeinschaft (DFG) Cluster of Excellence "Precision Medicine in Chronic Inflammation" focusing on clinical sequencing and the clinical utility of polygenic risk scores as well as its implication on precision medicine in the field of the inflammatory diseases inflammatory bowel disease, atopic dermatitis and coronary artery disease. Additionally, we highlight current developments and discuss challenges to be faced in the future. Exemplary, we point to residual challenges in detecting disease-relevant variants resulting from difficulties in the interpretation of candidate variants and their potential interactions. While polygenic risk scores represent promising tools for the stratification of patient groups, currently, polygenic risk scores are not accurate enough for clinical setting. Precision medicine, incorporating additional data from genomics, transcriptomics and proteomics experiments, may enable the identification of distinct disease pathogeneses. In the future, data-intensive biomedical innovation will hopefully lead to improved patient stratification for personalized medicine.

Keywords: inflammation, atopic dermatitis, inflammatory bowel disease, coronary artery disease, genome-wide association studies, polygenic risk score, whole-exome sequencing

INTRODUCTION

Since sequencing-based high throughput methods have led to cost-effective sequencing of big patient cohorts, our understanding of the genetic background of diseases has evolved. But the more data we are accumulating, the more we understand how complex the genetic background of some diseases is. In chronic inflammatory diseases, such as inflammatory bowel disease (IBD), atopic dermatitis (AD) and coronary artery disease (CAD), research has revealed a number of risk loci that are involved in disease pathophysiology. In spite of the growing number of identified genetic risk genes, functional targeted therapies evolving from our newfound genetic understanding are still in

their infancy. The reasons are as manifold as the genetic variants that can lead to complex inflammatory disease. Which variants lead to a phenotype? Which combinations of variations, but not single variants lead to a combined effect that causes physiological impairments? Are patient cohorts where genetic information is derived from predictive for individual patients? And even if we can pinpoint a causative variant, can patients profit from this?

With the rise of high-throughput methods in sequencing we stand on the brink of a revolution in precision medicine. We deepen our understanding of the genetic background that underlies disease on an individual basis and with this we, for the first time, have the tools to implement therapies that distinguish disease subtypes but likewise optimize drug efficacy and minimize side effects. Clinical sequencing for precision medicine can be applied on several levels. Primarily, sequencing provides basic information and with this a characterization of the genetic background of disease. On a second level, genetic information can lead to the generation of prospective knowledge of disease risk, disease severity and disease outcomes. Moreover, sequencing can lead to the identification of subtypes of the disease based on their genetical characteristics.

IBD, AD and CAD represent multifactorial disorders, with genetic as well as environmental factors contributing to the respective clinical phenotype. The complex genetics of these diseases has been comprehensively studied. However, our current understanding of their etiology is still limited. Various studies based on national health registries report an association between the diagnoses of AD and IBD, suggesting a shared pathophysiology. Indeed, e.g., increased TH1/TH17 signaling and the resulting secretion of proinflammatory cytokines represent mutual hallmarks of these diseases (1–3). Likewise, there is evidence for IBD patients to be at an increased risk of atherosclerosis and, consequently, an increased risk for cardiovascular diseases, including CAD. Postulated pathological links between the diseases are manyfold and include the deregulation of inflammatory mediators, dysfunction of endothelial barriers as well as effects of gut microbial endotoxins (4–7).

Abbreviations: AD, atopic dermatitis; ANGPTL4, angiopoietin-like 4; APOB, apolipoprotein B-100; ATG16L1, autophagy related 16 like 1; AUC, area under the ROC curve; CAD, coronary artery disease; CD, Crohn's disease; CD200R1, cell surface glycoprotein CD200 receptor 1; DOK2, docking protein 2; EDC, epidermal differentiation complex; eoIBD, early-onset IBD; FH, familial hypercholesteremia; FLG, Profilaggrin; FLG2, filaggrin-2; GPRS, genetic profile risk scores; GWAS, genome-wide association studies; HLA, Human leukocyte antigen; HLA-DRB1, HLA class II, histocompatibility antigen, DRB1 beta chain; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HSCT, hematopoetic stem cell transplant; IBD, inflammatory bowel disease; IBD-U, IBD unclassified; IL10R, interleukin 10 receptor; IL23R, interleukin-23 receptor; IRGM, immunity-related GTPase family M; LDLC, low density lipoprotein cholesterol; LDLR, LDL receptor; LRRC32, Leucine Rich Repeat Containing 32; MAF, minor allele frequency; MI, myocardial infarction; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NOD1/2, nucleotide-binding oligomerization domain-containing protein 1/2; PCSK9, proprotein convertase subtilisin/kexin type 9; PRS, polygenic risk score; RPTN, repetin; SNP, single nucleotide polymorphism; UC, ulcerative colitis; veoIBD, very-early-onset IBD; WES, whole-exome sequencing; WHO, world health organization; XIAP, X-linked inhibitor of apoptosis.

HIGH-THROUGHPUT SEQUENCING PROVIDES NEW INSIGHT ON THE GENETIC BACKGROUND OF INFLAMMATORY DISEASES

Trio Exome Sequencing Reveals Mono- and Oligogenic Forms of IBD

Inflammatory bowel diseases are chronic, relapsing disorders involving inflammation of the gastrointestinal tract caused by the interplay of an overly active immune system and environmental triggers in genetically susceptible individuals. The most common subforms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Hundreds of mostly common susceptibility variants have been identified through genome-wide association studies (GWAS), but there are also cases where rare, highly penetrant variants have a large impact on disease. Early-onset cases of IBD (eoIBD), with a disease manifestation during the first 10 years of life, often show a more severe disease course with a higher risk of complications. Furthermore, they are sometimes affected by genetically less complex (monogenic or oligogenic) types of the disease. For example, mutations in genes for the interleukin 10 receptor (IL10R) subunit proteins and the *IL10* gene itself have been shown to be responsible for several cases of severe eoIBD. Recently, we revealed compound-heterozygosity for a missense and a synonymous variant affecting splicing in IL10RA in one patient through trio exome sequencing (8). The *XIAP* (X-linked inhibitor of apoptosis) gene has been shown to be responsible for eoIBD in several male patients. We detected a hemizygous *de novo* nonsense mutation in one of our patients resulting in a selective defect in NOD1/2 signaling (nucleotide-binding oligomerization domain-containing proteins), impaired NOD1/2-mediated activation of NF-κB (nuclear factor "kappa-light-chain-enhancer" of activated B-cells) (9). We also showed a likely synergistic interaction between a rare hemizygous variant in the *NOX1* (NADPH oxidase) gene and a common homozygous variant in the *CYBA* ((Cytochrome B-245 Alpha Chain) gene altering its antibacterial activity in another veoIBD (very-early-onset IBD) patient (10). These examples illustrate the benefit of exome sequencing, and especially trio exome sequencing for diagnostics in eoIBD patients for the identification of *de novo* and compound-heterozygous variants and the reduction of candidate variants in general.

Association Studies Point to Roles for Common and Rare Variants in AD

Atopic dermatitis is a complex, polygenic, chronic cutaneous disorder. With a lifetime prevalence of up to 20% it represents the most common inflammatory disease of the skin. Atopic dermatitis is believed to be a cutaneous manifestation of a systemic disorder that also gives rise to other atopic conditions, such as asthma and allergic rhinitis. Current models assume a complex interaction between genetic, immunological and environmental factors to be involved in the etiopathogenesis of the disease. For further details, reference is made to recent reviews (11, 12).

A multitude of GWAS has been conducted to detect common variants related to the susceptibility for atopic dermatitis (13–19). In a summarizing meta-analysis Paternoster et al. identified ten novel risk loci, increasing the number of known loci to 31 (20). The most recent association study of rare protein-coding variants incorporating genetic data of as much as 15,574 patients and 377,839 controls resulted in the detection of *DOK2* (docking protein 2) and *CD200R1* (cell surface glycoprotein CD200 receptor 1) as additional susceptibility genes (14). Current estimates of heritability explained by common AD susceptibility variants (minor allele frequency MAF≥1%) amount to 14.91%. An additional 12.56% of heritability are estimated to be attributable to rare protein-coding variants (MAF<1%) (21).

Coding regions for major genes of the late epidermal differentiation have been identified to be colocalized within the so-called epidermal differentiation complex (EDC). Profilaggrin (*FLG*), filaggrin-2 (*FLG2*), and repetin (*RPTN*), represent a subset of EDC gene products contributing to the maturation of the human epidermis. Mutations of the *FLG* gene have been repeatedly shown to be associated with susceptibility and persistence of AD. However, this association could only be observed in individuals of European or Asian ancestry. Based on whole-exome sequencing (WES) mutations of *FLG2* (22, 23), *RPTN* (24), and *CLDN1* (Claudin 1) (25, 26) have been identified to be associated with susceptibility to AD in patients of non-European descent. This suggests factors causing dysfunction of the skin barrier vary across ethnicities (25, 26).

Immunological dysregulation represents another major factor contributing to the etiology of AD. Human leukocyte antigen (HLA) genes, such as *HLA-DRB1* (HLA class II histocompatibility antigen, DRB1 beta chain), play a crucial role for the presentation of antigens to the immune system and have been shown to be associated with the disease. Further immune abnormalities observed in AD and its common comorbidities are caused by mutations of the gene *LRRC32* (Leucine Rich Repeat Containing 32). Using a targeted sequencing approach our group identified and validated low-frequency variants of the gene as strong contributors to AD (27).

Heritability of CAD and MI Is Only Partially Explained by Currently Known Risk Alleles

Atherosclerotic vascular disease and particularly coronary artery disease remain leading causes of mortality worldwide. Atherosclerosis is initiated by lipid-mediated damage to the endothelium, followed by inflammatory cell recruitment and development of plaques, ultimately leading to plaque erosion or rupture as well as clinical sequelae such as myocardial infarction (MI) or stroke. The use of human genetics to reveal causal mechanisms has proved transformative for deriving aetiological insights in CAD beyond established concepts.

Rare variant analyses have provided examples on how genetic discoveries can point to therapeutic approaches for CAD, e.g., inhibition of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) (28), PCSK9 (proprotein convertase subtilisin/kexin type 9) (29), and ANGPTL4 (angiopoietin-like 4) (30). Genome-wide arrays preferentially contain single-nucleotide polymorphisms (SNPs) that are found at a high frequency in a population as those offer the highest statistical power to detect association. Accordingly, almost all currently identified 164 risk alleles for CAD are common (31). Given the large number and the high frequency of risk alleles that have been identified thus far, virtually every person in our population carries multiple genetic variants that increase susceptibility to coronary disease (32). Each risk allele increases the probability of CAD only by a relatively small margin, i.e., 5–20 relative percentage points per allele. There are two exemptions: One low frequency allele on chromosome 6q25.3 tags markedly increased lipoprotein levels and goes along with a risk increase for coronary disease by 54% (33). The other one is a relatively common variant on chromosome 9p21.3, which increases relative risk by 29% (34).

The rapidly growing list of genetic loci associated with increased risk of CAD is surprising in many aspects. Exemplary, the majority of them has neither been implied in the pathogenesis of the disease (35) nor linked to traditional risk factors (36). Interestingly, the genetic component reflected by common genetic variants cannot explain familial clustering of the disease as well. A positive family history rather appears to be mediated by rare deleterious mutations with a more profound effect (37–39). Not surprisingly, the heritability of CAD and MI is only partially explained by currently known risk alleles (35).

CLINICAL UTILITY OF POLYGENIC RISK SCORES

Association Between Polygenic Risk Scores and Subtypes of IBD

During the past 15 years, GWAS have led to the identification of more than 200 susceptibility loci for inflammatory bowel disease (31, 40). Chen et al. (41) utilized this data to perform a comprehensive comparison of four methods to predict the genetic risk of IBD. With an area under the ROC curve (AUC) of up to 0.78 and 0.70 for CD and UC, respectively, the Bayesian mixture model outperformed the other methods. While this accuracy is not sufficient for diagnostic use in a clinical setting, the authors were able to identify significant associations of higher risk scores with an elevated frequency of bowel resection, earlier disease onset and ileal disease localization. Similarly, Cutler et al. observed a statistically significant relationship between a polygenic liability score and age of onset in pediatric CD patients (42). Ananthakrishnan et al. employed similar methods and found an increasing genetic burden to be associated with earlier age of diagnosis and ileal involvement in CD patients (43). Likewise, a genetic risk score incorporating all known IBD risk alleles showed strong association with disease subphenotypes (44). Predictive models based on this genetic risk score were able to distinguish between colonic and ileal CD. In contrast to adult-onset IBD, veoIBD with an age of onset before the age of six, can be associated with a wide range of rare monogenic, or Mendelian, disorders, but only in a fraction of

patients. Serra et al. generated polygenic risk scores based on the effect-size estimates of SNPs significantly associated with adult-onset CD and UC and analyzed whether veoIBD patients with an age of onset under the age of six, harbor a higher load of risk alleles when compared to adult-onset IBD cases or population controls. The risk scores of veoIBD patients were significantly higher compared to those of the healthy controls. However, there was no significant difference between the veoIBD and adult-onset cases (45). In summary, current literature renders polygenic risk scores a promising tool for stratifying IBD patients with regard to age of onset as well as severity of the disease. Current research, including ongoing work of our group, tries to further improve the accuracy of PRS-based predictors to enable future application in a clinical setting.

PRS-Based Stratification by Disease Susceptibility and Disease Course of AD

Jansen et al. employed additive polygenic risk scores of varying complexity to investigate the putatively increased susceptibility of children diagnosed with cow's milk allergy (CMA) for common comorbidities, including asthma and AD (46). For AD the authors detected a decreased PRS independent of the employed model. PRS-based prediction of further clinical parameters has been examined in a recent study by Abuabara et al. (47). In populations of varying ethnicity the authors provide evidence for a PRS being highly predictive of AD. However, ancestry-related genetic effects do not independently explain disparities in disease prevalence and disease control between the demographic groups under investigation. Clark et al. investigated the relationship between a PRS and distinct developmental profiles of eczema, wheeze, and rhinitis identified using Bayesian machine learning methods (48). The authors provide evidence for differential association of the PRS across the entirety of developmental profiles, suggesting heterogeneous mechanisms underlying individual disease trajectories. In summary, first studies describe PRS as promising tools for the stratification of cohorts of AD patient with regard to their disease susceptibility and disease course. However, further studies are needed, to replicate these findings.

Estimating CAD Risk Using PRS

Being a polygenic disease with a substantial heritability, CAD is an attractive target for risk estimation based on the genetic background. Models for risk estimation have already been proposed and entered clinical routine, such as the *HeartScore* (49) and the *Framingham Risk Score* (50). However, these are mainly based on clinical variables. Previously, efforts have been made to improve existing models by the addition of scores based on individual genetic variants (51–55). The PRS used in these approaches were limited by considering only genetic variants for which an association with CAD had previously been established. Recently, this limitation has been abolished by genome-wide polygenic risk scores proposed by Khera et al. (56) and Inouye et al. (57). Using millions of genetic variants to predict the risk of CAD and other complex diseases, these methods outperform model incorporating conventional risk factors. This suggests genetic risk prediction to enable effective prevention strategies.

It is arguable that summarizing the genetic risk using an inherent assumption of linearity is too simple given the complex biological structure of common diseases. Further, estimating the weight of each variant by univariate association tests only neglects possible interactions between variants. Especially in the MHC region, variants can exhibit non-linear effects on diseases through interactions (58–62). To assess this question for CAD, Gola et al. (63) compared various methods from the field of machine learning (ML), which offer attractive algorithms to model non-linear effects, with a GPRS in a case-control data set of samples of European descent from the German population. It turned out that a simple GPRS outperformed all other algorithms under consideration by means of a nested cross-validation. However, the models differed greatly in the number of variants used. While the GPRS utilized ~50,000 variants, the non-linear models were much more sparse, utilizing approximately 1,300 to 10,500 variants. The sheer number of variants in GPRS is an aspect that should not be neglected for their clinical utility: 1. Practical aspects. Although whole genome sequencing becomes cheaper, processing of the data still requires huge computational resources. Traditional genotyping arrays provide a much cheaper and faster way to type variants. However, customary arrays cover about 4.5 million variants, much less than the 6.9 million variants used by Khera et al. 2. Replication. The probability that all variants used by proposed GPRS are available in independent datasets is almost zero. Thus, imputation or proxy variants are necessary, making exact replication of GPRS impossible. 3. Population bias. Using more and more variants to construct GPRS results in overly population specific models. It has already been shown that GPRS developed in individuals of European descent cannot readily be applied to other ethnic groups without taking into account the target population's structure (64). Yet, it is unknown whether the performance of a GPRS utilising millions of variants depends not only on ethnicity, but also on smaller genomic differences within an ethnicity or even population.

CLINICAL SEQUENCING AND ITS IMPLICATION ON PRECISION MEDICINE IN THE CLINICAL PRACTICE

Clinical Sequencing Directly Affects Treatment of eoIBD Patients

The most important factors underlying IBD pathogenesis can be summarized as genetics, environment, microbiome, and immunome (indicating the dysregulation of the immune response in the gut) (65). However, the genetic basis alone is extremely complex: The susceptibility variants identified mainly through GWAS explain only a fraction of the expected heritability and most risk loci contain several candidate genes. Only for selected loci, causal variants and genes have been identified in the respective region. The genetic data from

GWAS already show associations of some variants or genes with a certain subphenotype and therefore allow the prediction of disease susceptibility and clinical phenotype up to a certain degree. Today, the diagnosis of CD or UC patients still primarily depends on endoscopy or colonoscopy, however, there are also patients in whom CD and UC still cannot be clearly distinguished leading to the unsatisfactory diagnosis of IBD-U (IBD unclassified). Furthermore, IBD patients have an extremely variable disease course, so the expectations for precision medicine do not only include the improvement of diagnostic methods but also the prediction of the disease course and the optimal treatment strategy.

Predictive models based on the genetic risk score were able to distinguish colonic from ileal Crohn's disease (44). Additional risk scores based on the microbiome may be possible in the future (66) but are not advanced enough as of yet. A transcriptional risk score based on summary-level GWAS and expression quantitative trait locus (eQTL) data integrated with RNA-seq data showed promising results: It outperformed genetic risk scores for discriminating between CD patients and healthy controls and was also able to predict disease course over time in pediatric CD patients (67).

Early-onset forms of IBD highlight the exceptional potential for precision medicine, since the identified variants can be functionally characterized to understand the mechanisms and directly target the disturbed pathways through therapy to correct the consequences of the genetic defect. Thus, in a therapy-refractory IBD patient with a genetic defect in the *XIAP* (X-linked inhibitor of apoptosis) gene and in young children with defects of the IL10 pathway, hematopoetic stem cell transplants (HSCT) were curative (68–71). These examples show the significant progress that has been made towards precision medicine in IBD, but they also highlight the substantial challenges we are facing.

Clinical Sequencing Enables Monitoring of Established and Development of Novel Treatment Strategies

Traditional treatment approaches for atopic dermatitis include topical as well as systemic therapies. Progress in understanding the pathophysiology of the disease facilitated the development of novel targeted therapeutic options. One classical hallmark of AD is the elevated expression of inflammatory cytokines, including the interleukins IL-4 and IL-13, propagating the dysfunction of the epidermal barrier. The monoclonal antibody Dupilumab (Sanofi S.A.), targeting both cytokines represents the first biologic agent approved for the treatment of AD (72). In a recent study Guttman-Yassky et al. provide evidence for the drug progressively improving disease activity, suppressing markers of inflammation and reversing the typical epidermal abnormalities (73). TREATgermany, a non-interventional multicenter patient cohort study has been initiated to assess effectiveness and safety of Dupilumab in the long term. Data collected at follow-up visits of this ongoing study confirm high rates of response without serious side effects (74). Tralokinumab (LEO Pharma A/S) and Lebrikizumab (F. Hoffmann-La Roche AG) represent further emerging treatment options. Tralokinumab, prevents IL-13 from binding to IL-13Rα1 as well as IL-13Rα2 (75). Lebrikizumab, in turn, selectively targets IL-13 and interferes with the formation of the IL-13Rα1/IL-4Rα receptor signaling complex (76). In independent randomized, double-blind, placebo-controlled phase 2b trials in participants with moderate-to-severe AD both drugs showed clear improvements of AD symptoms and an acceptable safety and tolerability profile.

Genetic Screening for Familial Hypercholesteremia

Large-scale sequencing in a clinical setting is not widely established for CAD, however in case of familial hypercholesteremia (FH) genetic cascade screening is recommended by the World Health Organization (WHO). Untreated FH significantly increases the risk for atherosclerosis and premature CAD (77). Because of its high prevalence and risk of severe complications, it is the only cardiovascular disease recommended for population-based screening by the WHO (78). Current recommendation endorses lipid screening, however genetic testing is encouraged for family-based cascade screening. Genetic testing is also useful to separate heterozygous and homozygous cases, as well as to uncover potential precision medicine targets (77). FH is mainly caused by variants in genes coding for proteins affecting hepatic LDLC uptake including the LDL receptor (*LDLR*), in which most disease-causing variants are found, as well as apolipoprotein B-100 (*APOB*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*). Multiple studies document the preventive effect of intensive medical LDL-lowering at young age to prevent cardiovascular events (78). Therefore, it has been suggested that incidental detection of variants leading to FH should be communicated to the affected individual and the family (79). In fact, owing to the high frequency of FH, several guidelines recommend programs to systematically unravel variants and to facilitate medical treatment already at young age.

PERSPECTIVES

Clinical sequencing has the potential to reveal directly actionable genetic variants, thus also directly affecting treatment of the patient. For some findings, treatment options already exist, such as HSCT for defects in *XIAP* or the IL10 pathway and monoclonal antibodies for directly targeting imbalanced metabolic processes. Novel findings drive forward the development of new drugs by revealing previously unknown pharmaceutical targets.

Cases reported within this review point to remaining challenges in detecting disease-relevant variants (**Table 1**). For example, the interpretation of synonymous and noncoding variants is still difficult and can lead to false-negative results. A possible complex interplay of rare and common variants, e.g., the two variants in *NOX1* and *CYBA* (3) makes the interpretation of sequencing data even more difficult. Low coverage in genes of interest can lead to reduced detectability of disease-related variants and should therefore be considered with caution. Finally, the choice of public databases represents another crucial factor influencing the results of the analysis. Thus, it needs to be acknowledged that frequency databases may contain

TABLE 1 | Challenges and possible solutions.

Challenges	Possible solutions
Interpretation of synonymous and non-coding variants, complex interplay of common and rare variants	- Ongoing developments of analysis/prediction tools taking into account findings from large cohort sequencing studies - Multi-omics approach combining, e.g., genomic, methylomic, transcriptomic and proteomic data with the microbiome, immunome and exposome
Low coverage of relevant genomic regions	- Deeper sequencing facilitated by a further decrease of sequencing costs - Sequencing of exomes or genomes as a replacement for gene panels to include all potentially relevant genomic regions
Large amounts of data from exomes and genomes overwhelm most diagnostic laboratories	- Development of out-of-the-box infrastructural and bioinformatic solutions
Databases, such as ClinVar and HGMD, include questionable variant classifications	- Critical handling and questioning of provided classifications - Further expert curation of databases
Risk prediction for complex diseases complicated by heterogeneity of phenotypes and genetic architecture	- Evaluation of impact of model type and complexity on the performance of PRS - Ethnicity-specific large cohort studies for evaluation and optimization of PRS performance

This table summarizes challenges of clinical sequencing in the context of inflammatory diseases as identified throughout this review and presents possible solutions.

(future) patients of the disease under investigation. Databases like ClinVar and HGMD that try to classify variants may also include errors, so variants listed as benign may still be potentially pathogenic, as can be seen for the known Factor-V-Leiden variant that was classified as benign by one submitter in ClinVar. In summary, the greatest bottleneck for clinical sequencing is still the interpretation of data and various factors need to be kept in mind when using NGS data in diagnostics. In the future, deeper sequencing, made possible through further decreasing sequencing costs, novel analysis tools, and the ongoing improvement of variant databases will allow for the more widespread application of clinical sequencing.

The shift from gene panels to whole exomes is not yet complete. Exome or whole genome sequencing still poses a challenge for small diagnostic laboratories concerning infrastructural and bioinformatic requirements resulting from the comparatively large volume of generated data. However, the high potential of clinical sequencing is reflected by the increasing rate of solved cases, ending the "diagnostic odyssey" that many patients with rare disorders are facing. Even if the identified genetic cause is not located in a gene that can already be directly targeted in therapy and for which no drugs exist yet, these findings help drive forward the development of future drugs by revealing novel research targets.

While findings from GWAS and first WES studies lead to the detection of loci being independently associated to the diseases, their interplay has been barely investigated. Furthermore, it can be reasonably assumed that complementing genomic data generated using sequencing technology by other omics layers will help to achieve this objective. Due to their complexity, inflammatory diseases are considered ideal targets for systems biology approaches and integration of multi-omics data. Multi-layered analyses combining, e.g., genomic, epigenomic and transcriptomic data with the microbiome, immunome and exposome are ideally suited to reveal the complex biology underlying the diseases and to identify subphenotypes of the diseases. This knowledge can then be used to develop the ideal treatment, specifically tailored to the patient´s needs and disease characteristics (**Figure 1**).

While the multi-omics approach is a promising strategy, due to its complexity it is not yet feasible to be used in a clinical setting.

Likewise, risk prediction for complex inflammatory diseases is complicated by the heterogeneity of each disease's phenotype and genetic architecture. Current polygenic risk scores therefore do not yet meet the requirements for diagnosis in the clinical setting. For a number of complex diseases, risk scores are utilized in the stratification of patients in the setting of randomized clinical trials, with the results likely to find their way into clinical practice in the next decade. Thus, in the future, polygenic risk scores may enable patient stratification early on after diagnosis based on their genetic risk and allow for closer monitoring of patients with a high genetic risk that are more prone to stronger disease severity.

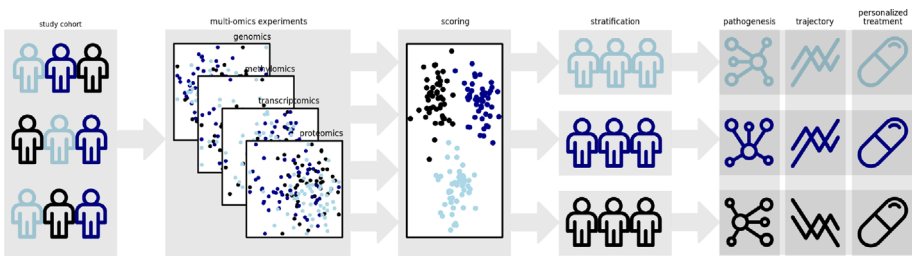

FIGURE 1 | Polygenic risk scores represent promising tools for the stratification of patient groups. Incorporation of additional data from methomics, transcriptomics and proteomics experiments might enable the derivation of multidimensional scoring schemes allowing a more accurate clustering of molecular disease phenotypes. The identification of these disease subtypes might enable the elucidation of distinct disease pathogeneses and trajectories. Ultimately, it will allow custom strategies for care and treatment of the individual patient.

AUTHOR CONTRIBUTIONS

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Macrophage-Targeted Lung Delivery of Dexamethasone Improves Pulmonary Fibrosis Therapy via Regulating the Immune Microenvironment

Xiaoqing Sang[1,2], *Yuanyuan Wang*[1,2], *Zhifeng Xue*[1,2], *Dawei Qi*[3], *Guanwei Fan*[4,5], *Fei Tian*[2,6], *Yan Zhu*[1,2] *and Jian Yang*[1,2]*

[1] Tianjin Key Laboratory of Chinese Medicine Pharmacology, Tianjin University of Traditional Chinese Medicine, Tianjin, China, [2] State Key Laboratory of Component-Based Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China, [3] Medcity Research Laboratory, University of Turku, Turku, Finland, [4] Medical Experiment Center, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China, [5] Tianjin Key Laboratory of Translational Research of Traditional Chinese Medicine Prescription and Syndrome, Tianjin, China, [6] Tianjin Key Laboratory of Traditional Chinese Medicine Chemistry and Analysis, Tianjin University of Traditional Chinese Medicine, Tianjin, China

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***Correspondence:**
Jian Yang
yang.j2017@tjutcm.edu.cn

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Idiopathic pulmonary fibrosis (IPF) is serious chronic lung disease with limited therapeutic approaches. Inflammation and immune disorders are considered as the main factors in the initiation and development of pulmonary fibrosis. Inspired by the key roles of macrophages during the processes of inflammation and immune disorders, here, we report a new method for direct drug delivery into the *in-situ* fibrotic tissue sites *in vitro* and *in vivo*. First, liposomes containing dexamethasone (Dex-L) are prepared and designed to entry into the macrophages in the early hours, forming the macrophages loaded Dex-L delivery system (Dex-L-MV). Chemokine and cytokine factors such as IL-6, IL-10, Arg-1 are measured to show the effect of Dex-L to the various subtypes of macrophages. Next, we mimic the inflammatory and anti-inflammatory microenvironment by co-culture of polarized/inactive macrophage and fibroblast cells to show the acute inflammation response of Dex-L-MV. Further, we confirm the targeted delivery of Dex-L-MV into the inflammatory sites *in vivo*, and surprisingly found that injected macrophage containing Dex can reduce the level of macrophage infiltration and expression of the markers of collagen deposition during the fibrotic stage, while causing little systematic toxicity. These data demonstrated the suitability and immune regulation effect of Dex-L-MV for the anti-pulmonary process. It is envisaged that these findings are a step forward toward endogenous immune targeting systems as a tool for clinical drug delivery.

Keywords: macrophages, pulmonary fibrosis, drug delivery, phenotypic regulation, immune microenvironment

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic interstitial pneumonia of unknown etiology. There is currently poor prognosis with increased morbidity and prevalence (1). Currently, only two clinical approved drugs, nintedanib (NDN) and pirfenidone (PFD) are being pursued to treat mild to moderate IPF by slowing down the disease progression (2). Yet,

these two drugs may easily cause side effects such as gastrointestinal reactions, liver dysfunction, photosensitivity and diarrhea, leading to the median survival time remains 2–5 years (3). To date, there is an urgent need to develop novel anti-fibrotic therapies capable of suppression of progression of IPF with minimal side effects.

The strategy exploiting immune systems for drug delivery is an area of great interest, as immune cells such as macrophages, neutrophils, and dendritic cells could sensitively respond to the complex inflammatory microenvironment at acute injury sites (4). These white blood cells can efficiently sense chemokines and cytokines factors and can be recruited into the inflamed tissues or/and sites (4, 5). Inflammation and immune disorders are considered as the main factors in the initiation and development of pulmonary fibrosis (6); in particular, alveolar macrophages or their prototype monocytes, especially can mediate and respond to the fibrosis related inflammatory stimulus or immune factors, such as interleukin 6 (IL-6), transforming growth factor-β (TGF-β), arginase-1 (Arg-1), etc. (7, 8). In addition, macrophages also can migrate to inflammation sites (9). These unique properties make macrophages a potential vehicle for pulmonary fibrosis drug delivery.

Depending on the local microenvironment on the fibrotic stage, macrophages can be replaced with classical activation (M1) and alternative activation (M2) phenotypes (10). In general, M1 type macrophages are responsible for wound healing after alveolar epithelial injury by secreting pro-inflammatory cytokines such as interleukin 6 (IL-6), promoting the elimination of foreign pathogens and inducing of inflammatory injury (11), while M2 type macrophages express transforming growth factor (TGF-β1), arginase-1 (Arg-1) and interleukin 10 (IL-10), which play the role of anti-inflammation, pro-angiogenesis and tissue repair (12). These anti-inflammatory cytokines also stimulate fibroblast proliferation and collagen production, which is important in the healing process, but increased expression of fibrotic factors, subsequently induced pulmonary fibrosis (13, 14). Both excessive M1 and M2 macrophages play dominant roles in IPF progression, making them promising targets via modulating macrophage polarization to reduce the level of fibrosis (15).

Exploiting macrophage for drug delivery causing anti-inflammatory response has previously been demonstrated in tissues other than the lung. Zheng et al. used tungsten oxide (WO) and indocyanine green (ICG) to construct nanoparticles. After macrophages swallowed the nanoparticles, the cells were injected through the tail vein to make the cells reach the tumor site and then given near-infrared irradiation which kill the engulfed tumor cells by photothermal effect (16). Zhang et al. designed a silica-based drug nanocapsule, which reached 16.6 pg of doxorubicin (Dox) loading per macrophage cell, allowing macrophages to reach the tumor without affecting the speed of cell migration. The drug is released after the site, which improves the utilization of the loaded drug (17).

It is worth noting that there are no reports of using macrophages as a delivery system for the treatment of pulmonary fibrosis. Moreover, no studies have reported the effect of drug-containing nanocarriers on the phenotypic changes of macrophages during the treatment of diseases. Here, we report a macrophage-mediated drug delivery system, which achieve efficient and accurate pulmonary drug delivery, with improved bioavailability of anti-pulmonary fibrosis drug dexamethasone (Dex) into the lung. Results showed that Dex decorated with liposomes (Dex-L) increased the loading efficiency of Dex into macrophages, similarly it had a bidirectional regulation effects to the phenotype when responding with the polarized macrophages which exacerbate the lung damage during the process of fibrosis. It was proved that Dex-L could keep the phenotype of the carrier itself in a balanced and non-pathogenic state, which also provided an explanation for the mechanism of Dex against pulmonary fibrosis. The therapeutic effects were further evaluated in a co-culture cell model of macrophages and fibroblasts and a mouse model of bleomycin-induced pulmonary fibrosis. We found that macrophages as a delivery system could effectively deliver Dex to the lungs with little systematic toxicity and exhibited excellent ability to inhibit pulmonary fibrosis. It is expected that this approach may provide an improved platform for targeted delivery of anti-fibrotic drugs while minimizing the problems of side effects.

MATERIALS AND METHODS

Materials

1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), dioleoyl phosphoethanolamine and (DOPE), cholesterol (CHO) were purchased from Avanti Lipid (Alabaster, AL, USA). All antibodies, including Alpha smooth muscle actin (α-SMA) mouse monoclonal, rat anti-F4/80 antibody, Rabbit anti-CD206 antibody, Alexa Fluor 488-conjugated goat anti-rabbit antibody, and Alexa Fluor 647-conjugated goat anti-rat antibody, were purchased from Abcam (Cambridge, MA, USA). BLM-A5 hydrochloride was purchased from Dalian Meilun Biotech (Dalian, China). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO USA). IL-4 was purchased from PeproTech (Rocky Hill, USA). TGF-β1 was purchased from R&D system. The ELISA test kit for IL-6, TGF-β1, IL-10, IL-1β, and Arg-1 were purchased from Zhuo Cai Biological Co., Ltd. (Shanghai, China). Nitric oxide synthase test kit was purchased from Biyuntian Co., Ltd. (Beijing, China). Masson's trichrome assay kit were purchased from Solarbio (Beijing, China). Sirius red reagent were purchased from Yuanye Bio-Technology (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Solarbio (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM) and other cell culture supplies were obtained from Gibco (Grand Island, NY, USA). Hoechst was provided by Invitrogen Co. (USA). Four percentage formalin was purchased from Weiao Biological (Shanghai, China).

Fabrication of Dexamethasone-Loaded Liposomes

Dissolved DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), cholesterol and DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) in a molar ratio of 2:1:1 in a mixed solvent of chloroform-methanol (1:1, v/v) and dexamethasone

dissolved in methanol with molar ratios of 1:1 in a 20 mL glass to form a film under nitrogen, respectively. Then the PBS (Phosphate Buffered Saline) was added to form a lipid suspension and ultrasonicated in a water bath at temperature 55°C for 30–40 min.

The Characterize of Dexamethasone Liposomes by Dynamic Light Scattering

The diameter distribution and zeta potential of different ratios of lipids and dexamethasone were determined by dynamic light scattering (DLS) using a Malvern Zeta Sizer Nano series (Malvern ZEN3600, Malvern, UK).

Cell Culture and Macrophage Polarization

Mouse macrophage RAW264.7 cell line and NIH-3T3 mouse fibroblast cell line were purchased from ATCC. Cells were maintained in Dulbeco's Modified Eagle's Medium (DMEM, Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco, 10099-141), 100 Units/mL penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 95% air with 5% CO_2. For the macrophage polarization, M1 macrophages were obtained by lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich Chemical Company) treatment for 12 h (18) and M2 macrophages were obtained by IL-4 (20 ng/mL, PeproTech) treatment for 48 h as previously described (19).

Toxicity Assay

The RAW264.7 cells were seeded into 96-well plates at a density of 3×10^4 cells/mL per well for 24 h before treatment and then incubated for another 24 h in the presence of different concentrations of Dex-L. The cell viability was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide]. After discarding the supernatant, each well was added 10% MTT and incubated for 4 h in the dark. The MTT solution was removed and then the formazan solution was added and shaking for 10 min. The absorbance was measured at 490 nm with a Microplate reader (Tecan, Groedig, Austria).

Determination of Drug Loading and Release From Macrophages

Dexamethasone was labeled with FITC (Fluorescein Isothiocyanate), a fluorescein and the drug loading of macrophages could be determined by the fluorescence intensity of FITC-labeled dexamethasone. Macrophages were incubated with FITC fluorescence-labeled dexamethasone liposomes and Triton X-100 was used to disrupt the cell supernatants at 0, 2, 4, 6, and 8 h. Following the fluorescence intensity of cell lysis solutions with different times were detected at excitation wavelength of 490 nm and emission wavelength of 515 nm. After macrophages were incubated with FITC fluorescence-labeled dexamethasone liposomes for 4 h, the liquid from the wells were removed. The phenol-free red blood cell culture medium containing 1% serum was added and the fluorescence intensity of supernatants at 0, 2, 4, 6, 8, and 10 h were tested under the same fluorescence detection conditions.

Co-culture Experiment

In our experiment, we used 24 mm Transwell® with 8 μm Pore Polyester Membrane Insert from Corning Company. The 3T3 cells were implanted in a 24-well plate. Before co-culture, macrophages and dexamethasone liposomes were allowed to interact 4 h in advance to complete the dexamethasone liposome payload. Then, the chamber was put into a 24-well co-culture plate and TGF-β1 (5 ng/mL) was added to the medium to induce the migration and activation of fibroblasts (20). Another co-culture method is collecting and resuspending the drug-loaded macrophages, they were added to a 24-well plate and co-cultured with 3T3 cells, and TGF-β1 was added to the medium to induce fibroblast activation (21).

Scratch Assay

Draw three straight lines after the 24-well plate, and insert the cells into the well plate at a suitable density. After the cells adhere to the wall, use a 200 μL pipette to make a scratch perpendicular to the three straight lines. Images were taken with an inverted microscope (LEICA DMi8, Germany) to record cell migration at 0, 24, and 48 h.

Immunofluorescence

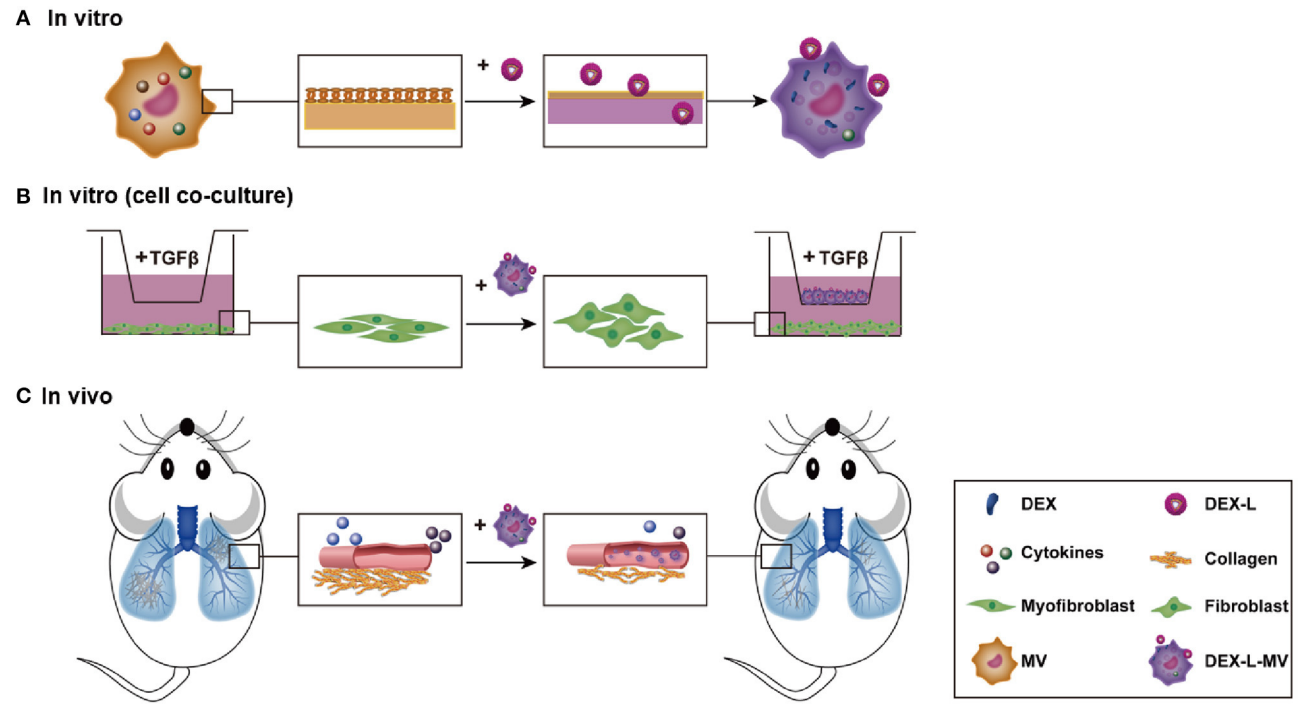
For immunofluorescence staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 min. Subsequently, the cells were treated with 2% BSA at 37°C for 1.5 h and incubated with the indicated antibodies at 4°C overnight. After being washed 3 times in PBS, the cells were stained with the corresponding secondary antibodies Alexa Fluor 488-conjugated rabbit anti-mouse antibody. Nuclei were stained with Hoechst 33342. Images were obtained with Operetta High Content Analysis (HCA) System (PerkinElmer, Boston, MA, USA). Quantitative analysis of fluorescence by ImageJ.

Fabrication of Nanoparticle-Laden Macrophages

Macrophages were incubated with dexamethasone liposomes for 4 h and the concentration of dexamethasone was 100 μM. The macrophages were gently scraped off with a cell scraper and resuspended in PBS. Then the cells were injected into mice via tail vein.

Animal Experiments

Idiopathic pulmonary fibrosis was established in mice model by intratracheal instillation of bleomycin (BLM)-A5 hydrochloride (Dalian Meilun Biotech, Dalian, China) (1.5 mg/kg dissolved in PBS). Mice were frequently examined by micro-CT after the treatment of BLM for 5th days. Mice presenting fibrosis lesions involving more than 25% of the lung were further divided into five experiment groups (6 mice per group), treating once daily with 3 mg/kg Dex (National Institutes for Food and Drug Control, Beijing, China). All the mice were administered via intravenous injection. Micro-CT scans were performed at baseline, then to evaluate the effect of these treatments. Lung tissues were quartered and processed for the following experiments: the left lobe was inflated and fixed in 10% buffered formalin for histological and immunohistochemical



SCHEME 1 | Drug delivery by macrophage targeted inflammatory microenvironment *in vitro* (**A**, cell level, **B**: co-culture level) and *in vivo* **(C)**.

examination, and the remaining lobes were stored at −80°C and used for preparation of whole lung tissue protein extracts.

Enzyme-Linked Immunosorbent Assay (ELISA)

The cell supernatant and mouse serum were centrifuged at 3,000 rpm for 10 min to be collected. The levels of TGF-β1 and IL-6 in the mice serum and cell-free supernatants were measured using the ELISA kits (Shanghai, China) according to the manufacturer's instructions and the cytokine concentrations were calculated using standard curves.

Mice lung tissues (50 mg) were homogenized, centrifuged and the supernatant was collected. Blood was collected from eyeball venous plexus of mice, and the serum stored at 4°C. IL-6, TGF-β1, IL-1β, and Arg-1 were detected by ELISA kits (Shanghai, China) according to the manufacturer's instructions.

Inflammatory Cell Analysis in Whole Blood

The whole blood was obtained from the eye orbit of mice and stored in an anticoagulant tube containing EDTA. Neutrophils and lymphocytes were detected using a blood analyzer (Premier 3000, USA).

Bronchoalveolar Lavage Fluid Collection

Mice were anesthetized by Tribromoethanol; a small incision was made on the trachea and BAL fluid was collected by cannula. Briefly, cannula was inserted into trachea with sufficient ice-cold PBS (0.5 mL each time) to collect BALF. The procedure was repeated thrice, and 70–80% recovery of collected BALF was observed. After collection of BALF, Later, BAL fluid was centrifuged at 4,000 rpm for 10 min at 4°C, and supernatant was used for determination of IL-6, TGF-β1, and IL-1β levels in BALF were detected by ELISA.

Immunohistochemistry, Hematoxylin-Eosin Staining (H&E), and Masson's Trichrome Stain

Immunohistochemistry was performed on 4 μm, paraffin embedded lung tissue and mounted on polylysine-coated slides. The slides were cleared of paraffin and subjected to antigen retrieval (10.2 mM sodium citrate, 0.05% Tween 20, pH 6.0, 10 min). Next, quenching of endogenous peroxidase activity was achieved by incubation with 3% (v/v) H_2O_2 for 10 min, followed by incubation with rabbit anti-CD206 at 4°C overnight. In addition, the slides were stained with H&E for structured observation, or with Masson's trichrome stain for detection of collagen deposits according to the instructions by the manufacturer.

Measurement of Hydroxyproline

The measurement of hydroxyproline was conducted with a hydroxyproline measurement kit (NanJing JianCheng Bioengineering Institute) according to the manufacturer's instructions. Approximately 30 mg (wet weight) lung tissue was collected. One milliliter of alkaline hydrolysate was added and the tissue was boiled at 95°C for 20 min with constant mixing. The pH was adjusted to 6.0–6.8 using the reagent provided. About 3–4 mL of supernatant was collected for

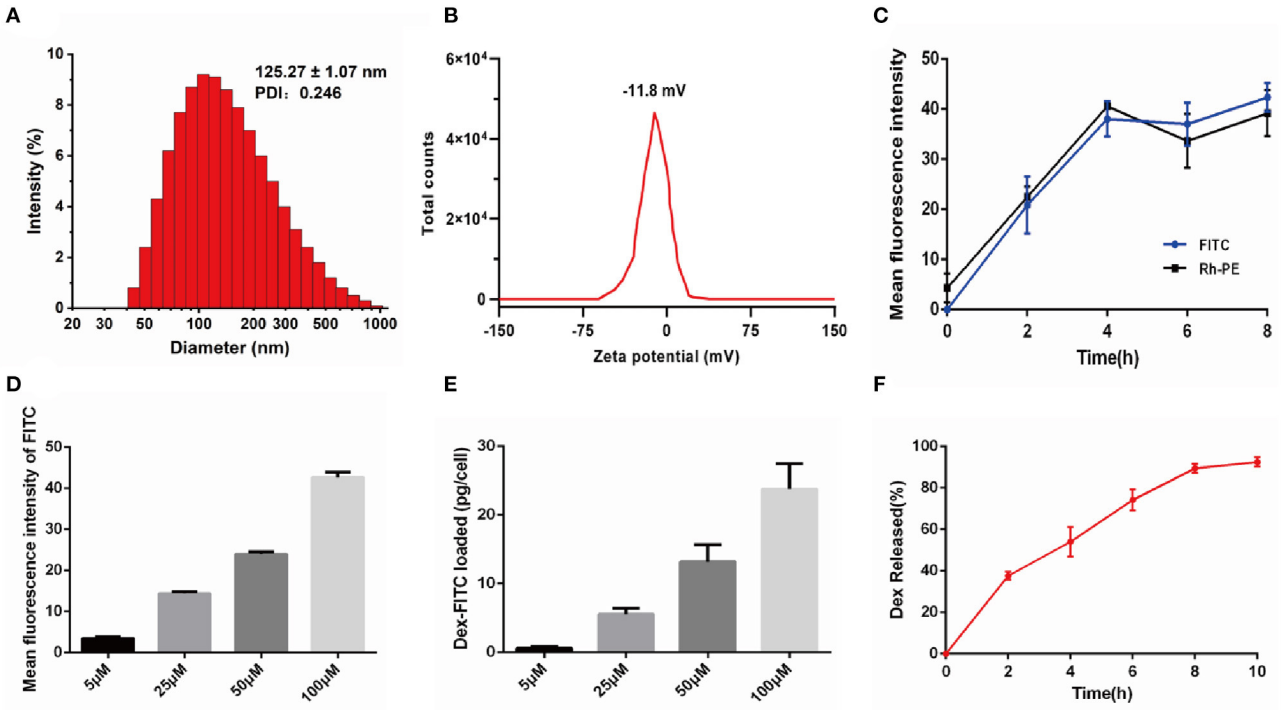


FIGURE 1 | Characterization and preparation of Dex-L and Dex-L-MVs. **(A)** The size distribution and **(B)** Zeta potential of the Dex-L measured by dynamic light scattering. **(C)** The uptake of Dex-FITC at a concentration of 100 μM after different incubation times with macrophages. **(D)** Mean fluorescence intensity of FITC fluorescently labeled Dex at different concentrations after incubating for 4 h with macrophages. **(E)** Intracellular Dex-FITC contents measured when the initial FITC fluorescently labeled Dex at the concentrations of 5, 25, 50, and 100 μM. **(F)** Percentage of Dex-FITC released from Dex-L-MVs at different time points. Mean values of three independent experiments with $n = 6$ cultures per group and experiment.

measurement after sorption onto active carbon. The hydrolysate was centrifuged at 3,500 rpm for 10 min. One milliliter of supernatant was then carefully taken for measurement according to the manufacturer's instructions.

Statistical Analysis

All analyses were performed using SPSS 7.0 software (SPSS Inc., Chicago, IL, United States) or GraphPad (GraphPad Prism 5, San Diego, CA, United States). The results were expressed as the mean ± standard deviation. Multi-group comparisons of the means were carried out by one-way analysis of variance (ANOVA) test with *post-hoc* Tukey's-test. The statistical significance for all tests was set at *P*-values of <0.05.

RESULTS

Preparation and Characterization of Dex-L-MVs

Our objective is to use macrophage loaded drug-liposome to target the inflammatory micro-environment in the pulmonary fibrosis and release the drug *in situ* (**Scheme 1**). First, the prepared dexamethasone liposomes (Dex-L) were taken-up by macrophages in the first few hours, forming the macrophage delivery system (Dex-L-MVs). To evaluate the effect of Dex-L to the inflammatory environment, Dex-L were incubated with polarized macrophages including M1 and M2 phenotype macrophages (**Scheme 1A**), Dex-L-MVs further seeded with TGFβ1-induced fibroblasts to show its affect during the cell interactions and activation process (**Scheme 1B**). Moreover, Dex-L-MVs as a drug delivery system were injected into bleomycin-induced pulmonary fibrosis mouse model via the tail vein, and evaluated for how it regulates the development of IPF and affects the immune microenvironment (**Scheme 1C**).

Liposomes encapsulated dexamethasone (Dex-L) was swallowed and prepared by the thin film dispersion method, the particle size of Dex-L measured by dynamic light scattering is 125.27 ± 1.07 nm (**Figure 1A**), and The ζ-potential of the Dex-L under physiological conditions was determined as −11.96 ± 0.28 mV (**Figure 1B**). To address the potential toxicity of Dex-L toward macrophages, cell viability was determined in RAW264.7 cells. MTT results showed that RAW264.7 cells well-tolerated Dex-L up to 100 μM after 24 h incubation (**Supplementary Figure 1**).

Next, in order to measure the drug loading efficacy, RAW264.7 cells were treated by the FITC fluorescence-labeled dexamethasone (f-Dex) and Rh-PE fluorescence-labeled

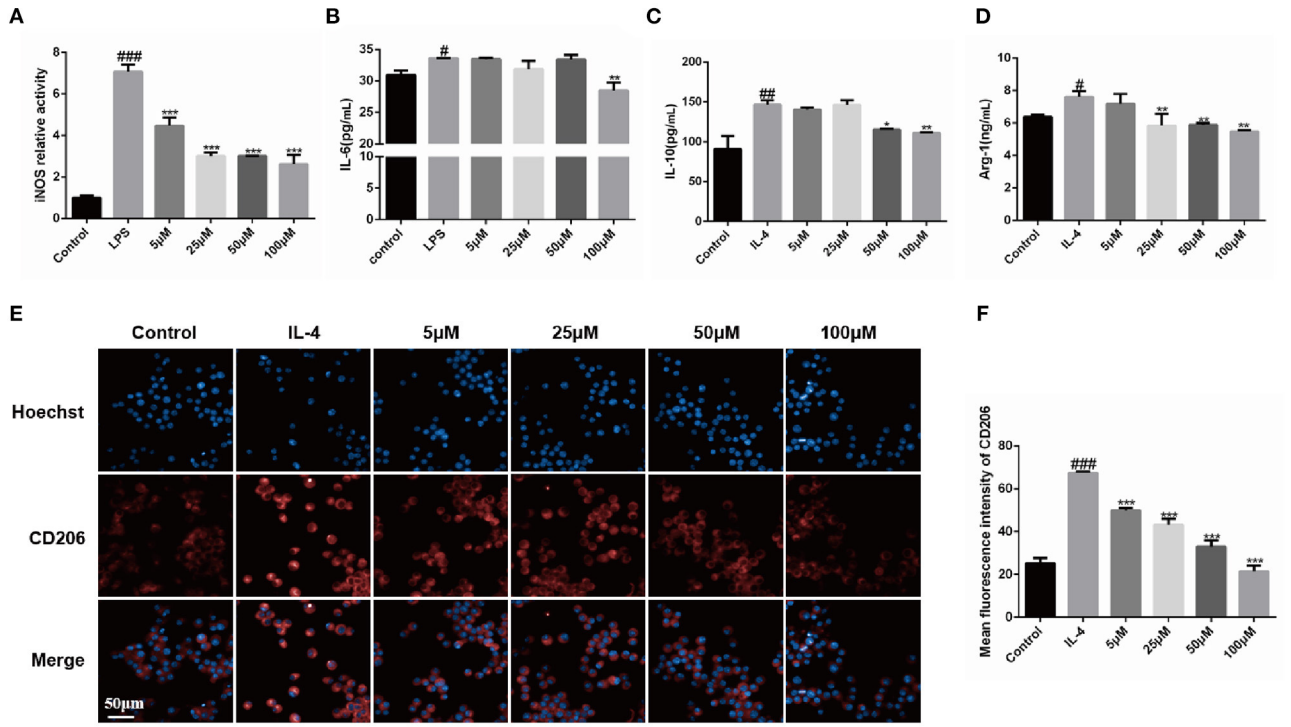


FIGURE 2 | Bidirectional regulation functions of Dex-L on polarized macrophage phenotypes. The iNOS **(A)** and IL-6 activity **(B)** of LPS-induced macrophage was down-regulated by Dex-L. The secretion of IL-10 **(C)** and Arg-1 **(D)** induced by IL-4 were decreased by Dex-L. **(E,F)** The expression and the qualification of fluorescence of CD206 in IL-4 induced macrophage. Scale bar = 50 μm. Values shown are mean ± S.D. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. control group, $^{*}p < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, vs. Model group. Mean values of three independent experiments with $n = 6$ cultures per group and experiment.

liposomes for different times. After measuring the fluorescence intensity of FITC and Rh-PE by Operetta High Content Analysis (HCA) System, we found that macrophages were incubated with f-Dex-L for 4 h leading to the highest effective intracellular uptake of Dex, while prolonged incubation time only slightly increased uptake of Dex (**Figure 1C** and **Supplementary Figure 2**).

The macrophages were incubated with liposomes containing f-Dex at different concentrations and the drug uptake of macrophages was concentration dependent (**Figure 1D** and **Supplementary Figure 3**). The uptake of macrophages at 4 h was about 2, 6, 13, and 25 pg/cell with the f-Dex-L at the dosing concentrations of 5, 25, 50, 100 μM, respectively (**Figure 1E**). Subsequently, f-Dex at the concentration of 100 μM released into macrophages was detected. Result showed that Dex released from macrophages was increased in a time-dependent manner reaching nearly 90% within 10 h (**Figure 1F**).

Dex-L Bidirectionally Regulates the Macrophages Polarization

Polarized macrophages (M1 and M2 macrophages phenotypes) play important roles during the development of fibrosis. To investigate the effects of Dex-L on the polarization status of macrophages, Dex-L were incubated with M1 and M2 macrophage and subsequently cytokine cues such as nitric oxide synthase (iNOS), IL-6 and IL-10 were determined.

As shown in **Figures 2A,B**, iNOS and IL-6 activities of macrophages in the group treated with 100 μM Dex-L was reduced by 63% (2.63 ± 0.45, $p < 0.001$) and 15% (28.5 ± 1.30, $p < 0.05$) respectively, indicating the M1 polarization was inhibited. **Figures 2C,D** shows the secretion of IL-10 and Arg-1 was also inhibited by Dex-L in a concentration-dependent manner. The expression of IL-10 and Arg-1 in the Dex-L administration group of 50 μM were down-regulated by 21.3% (115.30 ± 0.91, $p < 0.01$) and 22.6% (5.89 ± 0.10, $P < 0.05$) respectively. **Figures 2E,F** furtherly showed that quantitative fluorescence results of CD206 in the Dex-L administration group of 100 μM was 3-folds less than the M2 polarized group (67.40 ± 0.58 vs. 21.52 ± 2.64, $p < 0.001$). The liposomes without drug at a concentration of 100 μM sharply reduced the expression of CD206 in macrophages, indicating the inhibition effect during the process of M2 macrophage polarization (**Supplementary Figure 4**). All these striking results demonstrated the bidirectionally regulation functions of Dex-L on polarized macrophages, which reflecting in the down-regulation of pro-inflammatory and anti-inflammatory

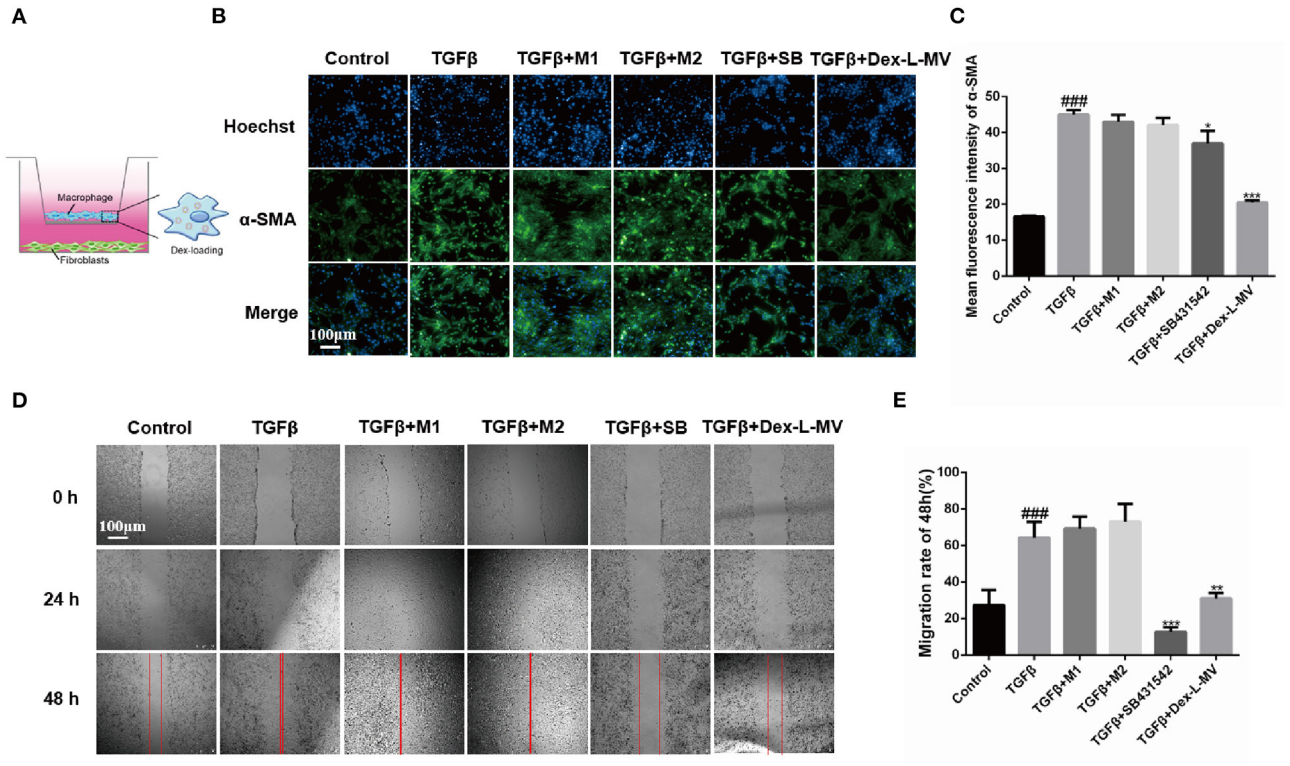


FIGURE 3 | Effect of Dex-L-MVs on TGF-β1-induced NIH-3T3 in co-culture system. **(A)** A schematic of co-culture system in Transwell. **(B)** and **(C)** Expression and the qualification of α-SMA of fibroblasts with Dex-L-MVs. Scale bar = 100 μm. **(D,E)** Inhibition of migration and the qualification of fibroblasts by Dex-L-MVs. Values shown are mean ± S.D. $^{###}p < 0.001$, vs. control group, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. Model group. All experiments were performed in triplicate, each being repeated at least three times.

factors, simultaneously preserving a balance in the secretion of cytokines. The special regulation could exert a positive effect in the progress of fibrosis.

Dex-L-MVs Inhibit the Migration and Activation of Fibroblasts in a Co-culture System

In order to evaluate the anti-fibrotic effect of Dex-L-MVs, we constructed a co-culture system of NIH-3T3 murine fibroblast cells and RAW264.7 macrophages (21). During the process of pulmonary fibrosis, the accumulation and activation of fibroblasts into myofibroblasts is largely responsible for the collagen production within alveolar structures (22). Here, NIH-3T3 fibroblasts were seeded in the lower chamber of a 24-well plate and the macrophages pre-loaded with Dex-L were co-cultured in the migration chamber (**Figure 3A**). As shown in **Figures 3B,C**, α-SMA, the activation marker of fibroblasts into myofibroblasts, was evaluated by immunofluorescence assay. Compared to activated fibroblast cells treated with TGF-β1, Dex-L-MVs showed even better inhibition of fibroblast activation (20.50 ± 0.55, $p < 0.001$) than the group treated with TGF-β1 inhibitor SB431542 (36.96 ± 3.56, $p < 0.05$). Similarly, the migration assay showed that Dex-L-MVs were capable of significant inhibition rate comparing to activated fibroblast cells (**Figures 3D,E**), where M1 and M2 macrophages stimulated the migration and activation of fibroblast cells (**Supplementary Figures 5, 6**). A 24-h migration result also concluded that M1 and M2 macrophages promoted fibroblast migration within 24 h, and it is stronger than the group induced by TGF-β1 alone (**Supplementary Figure 7**).

Altogether, co-culture results indicated that Dex-L-MVs had a good ability to inhibit the migration and activation of fibroblasts, further hindering the development of IPF.

Targeted Delivery of Dex-L-MVs Into Inflammatory Lung *in vivo*

A mouse model of pulmonary fibrosis was established by tracheal instillation of BLM (1.5 mg/kg) (23). We detected microCT scan imaging to allow the evaluation of lung fibrosis at different time points (0, 5, 10, 15 days) after bleomycin induction, and found that there were diffuse shadows in the lungs from the 5th day (**Supplementary Figure 8**). Therefore, after BLM induction on the 5th day, mice with more than 25% pulmonary fibrosis lesions were further divided into five experimental groups for the next drug treatment. The mice were sacrificed by excessive anesthesia at 15 days after BLM challenge (**Figure 4A**). The mean body weights of the mice in control group were slightly increased, while the weight

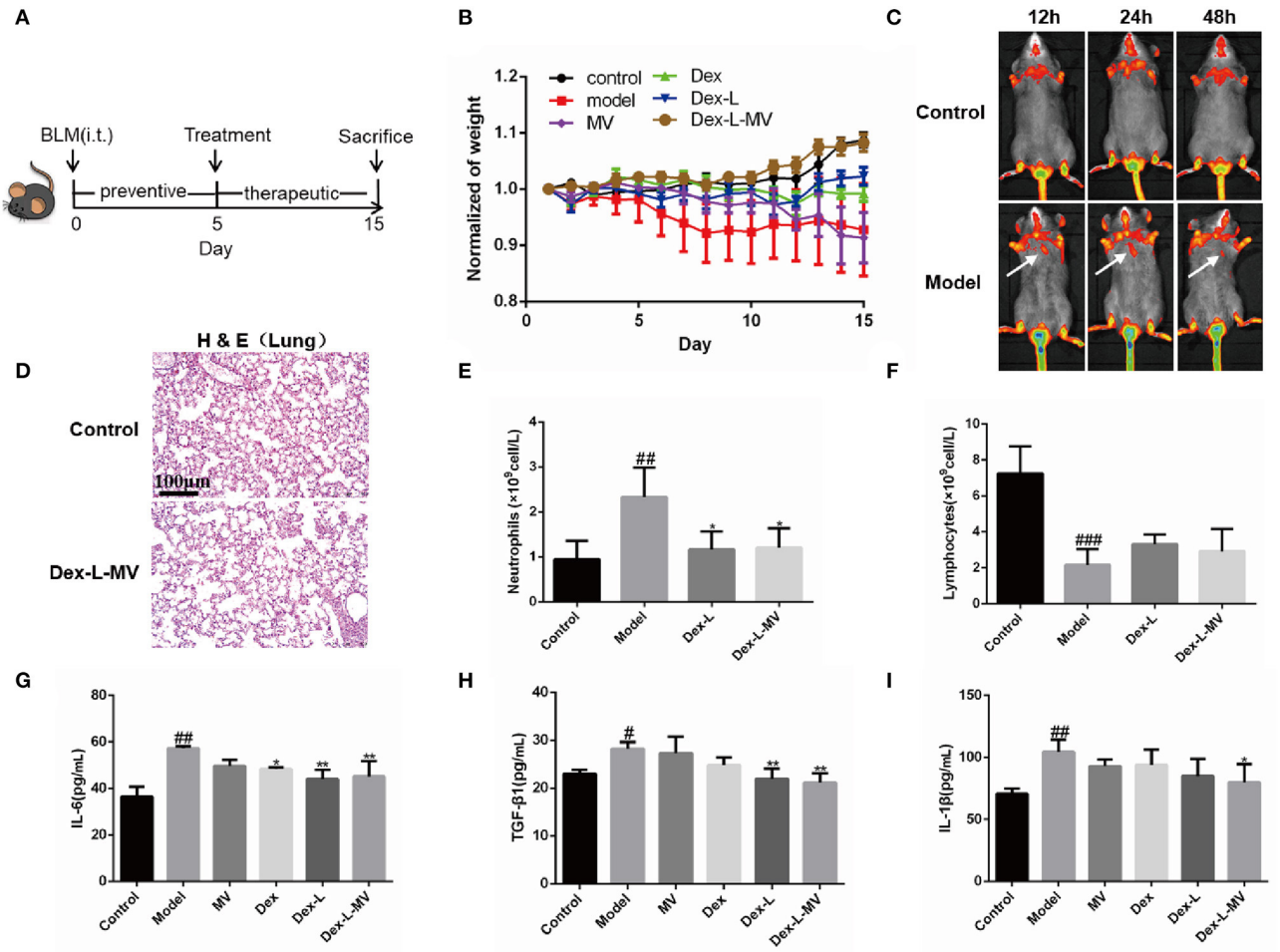


FIGURE 4 | Targeted delivery of Dex-L-MVs into inflammatory lung *in vivo*. **(A)** The timeline of administration with Dex-L-MVs. **(B)** The weight change of mice ($n = 6$). **(C)** The fluorescence intensity of lipid-labeled macrophages in lung of mice over the time after intravenous injection with Dex-L-MVs. **(D)** the lung systemic toxicity of Dex-L-MVs. Scale bar = 100 μm. **(E)** The number of neutrophils and **(F)** the number of lymphocytes neutrophils per liter of peripheral blood **(G–I)** The secretion levels of inflammatory factors including IL-6 **(G)**, TGF-β **(H)** and IL-1β **(I)** in mouse BALF on the 10th day. Data representative for three independent experiments, mean ± S.D. of at least four technical replicates per time point. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. control group, $^{*}p < 0.05$; $^{**}p < 0.01$, vs. Model group.

of mice instilled with BLM significantly decreased on 5th day. **Figure 4B** showed that the mice administrated with Dex, Dex-L, and MVs showed weight restore, while the group with Dex-L-MVs markedly reversed these reductions. To test the delivery positions of Dex-L-MVs, we labeled liposomes with Liss Rhod-PE (Rh-PE) red fluorescence and injected macrophages that phagocytosed fluorescent liposomes into mice. We observed the fluorescence accumulation in control mice and fibrotic mice after 12 h post-treatments. As expected, stronger fluorescence accumulation (white arrows) into the pulmonary of fibrotic mice when compared with that in mice in control group, indicating that Dex-L can more efficiently reach the lungs with the help of macrophages (**Figure 4C**). This result proved that the inflammatory pulmonary can recruit macrophages to achieve the targeted therapeutic effect. We investigated the systemic toxicity of Dex-L-MVs toward C57 mice ($n = 6$) using hematoxylin and eosin (H&E) staining. The results showed that no pathological changes in organs after 10 days treatment, demonstrating that systemic toxicity was rarely exhibited by intravenous injection of the Dex-L-MVs groups (**Figure 4D** and **Supplementary Figure 9**). Next, we further investigated the effect of dexamethasone-liposomes infused into the lungs of mice on the regulation of immune responses. As expected, the blood routine results showed that the model group mice had up-regulation of neutrophils and down-regulation of lymphocytes, when compared to the wild-type mice. After the administration of Dex-L-MVs, the increase of neutrophils and the decrease of lymphocytes were significantly inhibited (**Figures 4E,F**). Moreover, after detecting the expression of inflammatory cytokines and chemokines in the collected bronchoalveolar lavage fluid, the results demonstrated that the inflammatory factors including IL-6,

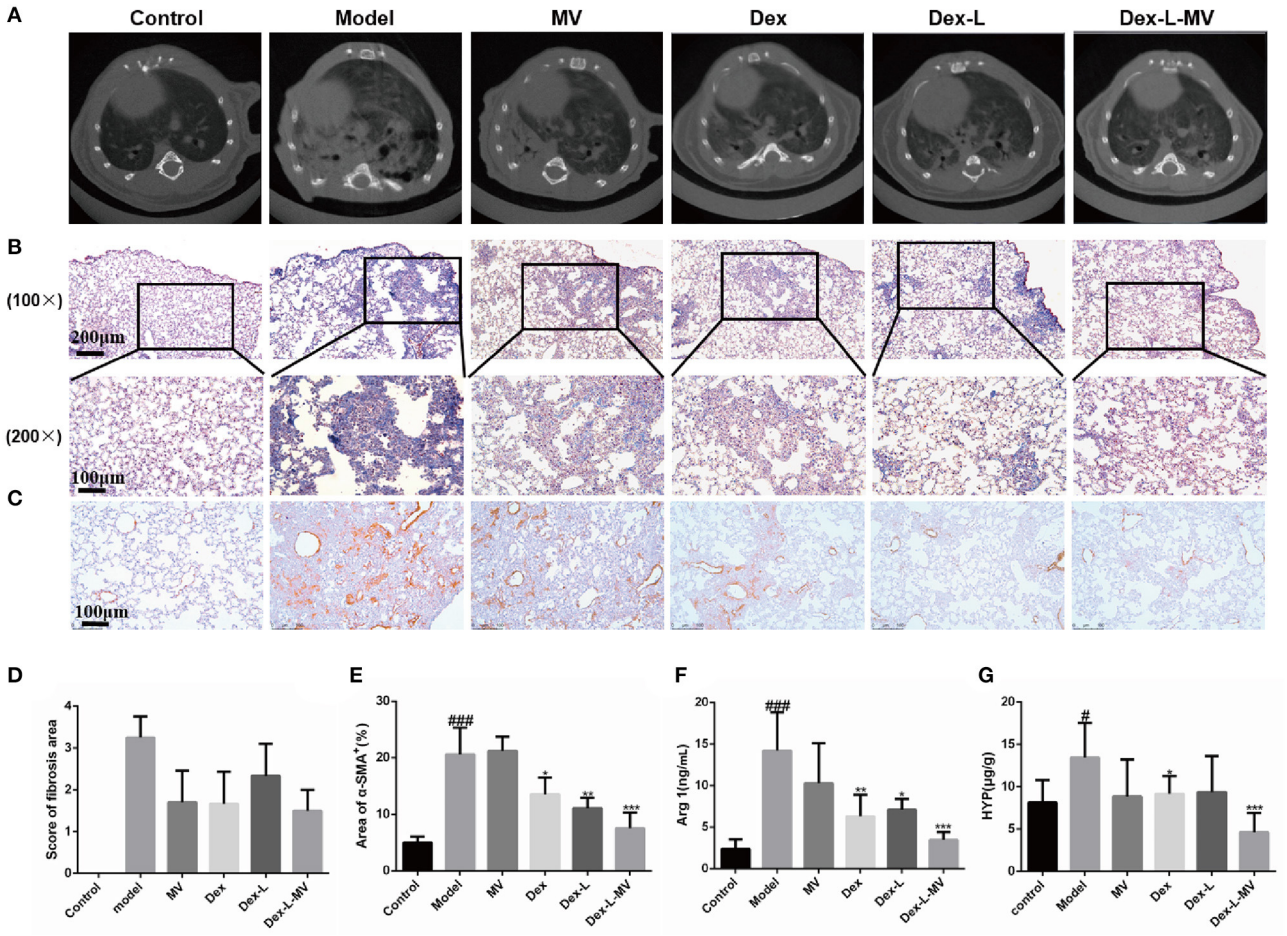


FIGURE 5 | Therapeutic effects of Dex-L-MVs on bleomycin-induced mouse model of IPF. **(A)** μCT scanning images to show the pulmonary fibrotic level of different groups. **(B)** The evaluation qualification of collagen deposition by Masson's trichrome staining of lung tissue sections. The inset shows a 4x image of lung lobe **(C)** The evaluation and **(E)** qualification of α-SMA by Immunohistochemistry of lung tissue sections. **(D)** The score of μCT images according to 85% percentile density. **(F)** The content of Arg-1 expressed in lung tissues. **(G)** The content of HYP expressed in lung tissues. All experiments were performed in triplicate, each being repeated at least three times. Values shown are mean ± S.D. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, vs. control group, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. Model group.

TGF-β1, and IL-1β were significantly decreased after the administration of Dex-L and Dex-L-MVs, indicating the abnormal fibrosis inflammatory microenvironment was well-remodeled (**Figures 4G–I**). Together, these results indicate the potential application of Dex-L-MVs induced targeted delivery for direct regulation of inflammatory and immune responses.

Therapeutic Effect of Dex-L-MVs on Bleomycin-induced IPF Mice

A usual interstitial pneumonia pattern on high-resolution computed tomography (HRCT) is essentially diagnostic of IPF in the appropriate clinical setting. Recently micro-CT (μCT) has been used to quantify the pulmonary fibrosis in mice, and the image quality of the μCT of each mouse was assessed semi-quantitatively on a four-point ranking scale as previously described (24, 25). The μCT results in **Figures 5A,D** showed that mice treated with Dex-L-MVs reduced the degree of BLM-induced fibrosis, with a score of 1.5–2. Nevertheless, model group and MVs group showed increasing obscured pulmonary vessels and an abnormal bronchial wall contour, which mean ranking scale was defined around 4, the score of mice administrated with Dex or Dex-L was 2.5–3, which showed that the level of lung fibrosis was slightly reduced. Furthermore, Masso's trichrome staining in **Figure 5B** and **Supplementary Figure 10** demonstrated that collagen deposition in model group was more than 3-folds than that in control group and the Dex-L-MVs treatment group, indicating the significant inhibition effects of Dex-L-MVs on collagen deposition in the mouse model of IPF. To investigate the effect of the drug delivery system on the activation of pulmonary fibrosis effector cell myofibroblasts, we performed immunohistochemical staining on mouse lung tissue sections and detected the expression of α-SMA, a fibroblast activation marker. The results showed that α-SMA expressed in the Dex-L-MVs group was about 3 folds lower than that in the model group (7.53 ± 2.77 vs. 20.57 ± 4.76, $p < 0.001$) (**Figures 5C,E**). In addition, we also detected the expression

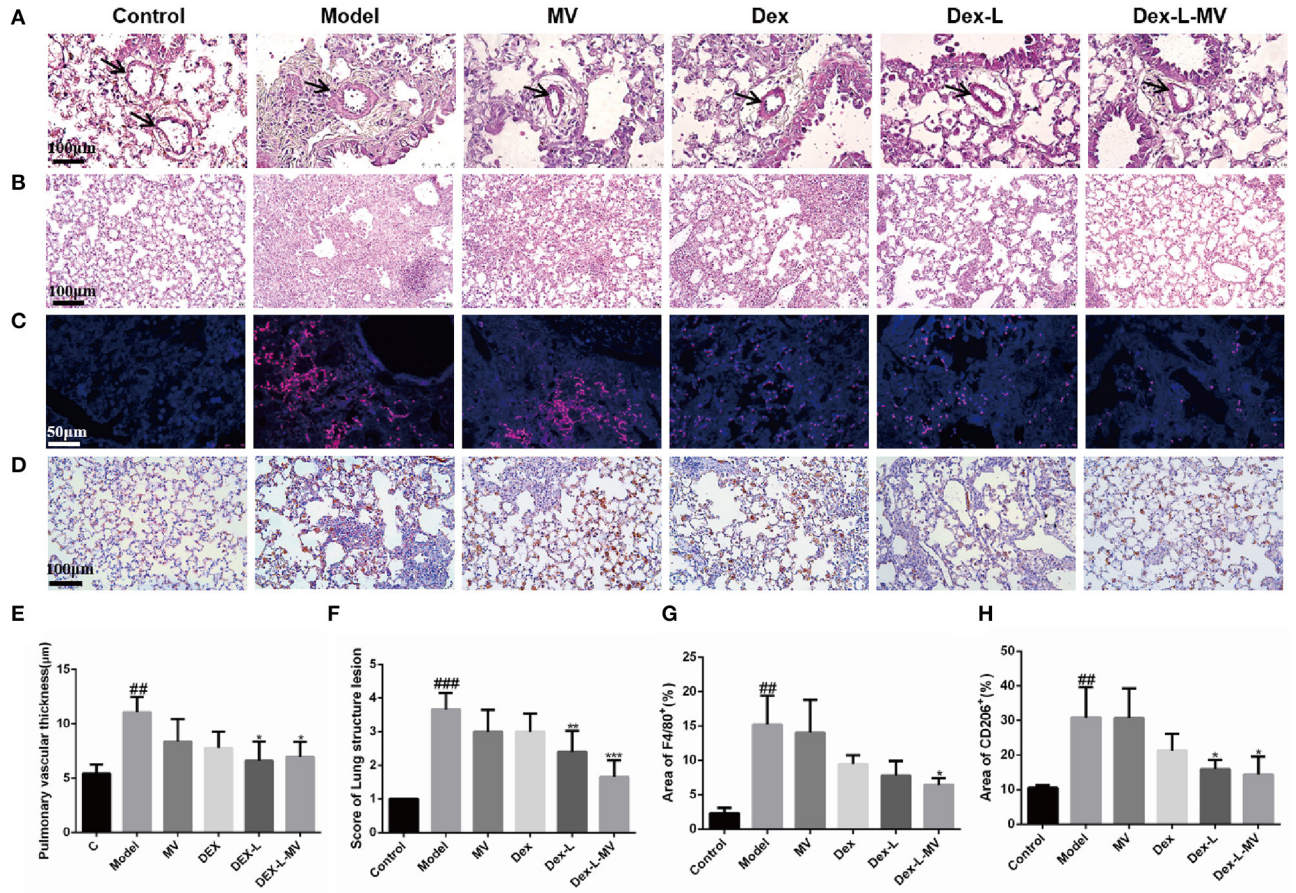


FIGURE 6 | The reduction of lung inflammation in fibrotic mice with Dex-L-MVs. **(A,E)** The thickness changes of lung vascular wall with H&E staining after administration and qualification by ImageJ. Scale bar: 100 μm. **(B,F)** The inflammation and the alveolar structure of lung with H&E staining and qualification analyses. Scale bar: 100 μm. **(C,G)** The macrophage labeled with F4/80 (red channel) in lung tissue using immunofluorescence. Scale bar: 50 μm. **(D)** and **(H)** The M2 macrophage labeled by CD206 with immunohistochemistry. Scale bar: 100 μm. All experiments were performed in triplicate, each being repeated at least three times. Values shown are Representative images mean ± S.D. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. control group, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. Model group.

of arginine-1 (Arg-1) in lung tissue homogenate (**Figure 5F**). The expression level of Arg-1 in the mice from the model group was increased around 4-folds when compared with that in control group (14.22 ± 4.59 vs. 2.37 ± 1.17, $p < 0.001$), after treatment with Dex-L-MVs, the expression of Arg-1 was significantly decreased (3.49 ± 0.93 vs. 14.22 ± 4.59, $p < 0.001$), which further presented the immune-modulatory effect of our macrophage delivery system containing DEX. We also evaluated the content of hydroxyproline (HYP) in the lung tissues, and found that Dex-L-MVs sharply reduced the amount of collagen in the lungs of BLM treated mice (**Figure 5G**).

Dex-L-MVs Reduces Lung Inflammation in Fibrotic Mice

During the development of fibrosis, the inflammatory response will drive the process of fibrosis (4). H&E stain of lung tissue revealed that severe pulmonary fibrosis was accompanied by a significant thickening of the blood vessel wall in the lung, which was suppressed by Dex-L and Dex-L-MVs treatment (**Figures 6A,E**). Mice in the model group showed severe inflammatory infiltration and destroyed alveolar structure. After Dex-L-MVs treatment, the lung inflammation in the mice was significantly reduced and the alveolar structure was significantly improved (**Figures 6B,F**). The occurrence of fibrosis is accompanied by large number of macrophages infiltration, especially the M2 type macrophage. In order to verify the regulatory effect of Dex-L-MVs on lung macrophage phenotype, we performed immunofluorescence staining and immunohistochemical staining on mouse lung tissue sections. Here, F4/80 was used to mark the total macrophages; the type 2 macrophages were labeled with CD206. Interestingly, Dex-L-MVs treatment not only down-regulated the total macrophages infiltration in the lung tissues of the mouse model of IPF (**Figures 6C,G**), but also reduced the total quantity of M2 macrophages which were marked by CD206 antibody (**Figures 6D,H**). These results indicate that Dex-L-MVs can improve lung fibrosis by reducing the activation of type 2 macrophages. ELISA results further indicated

the expression levels of IL-6, TGF-β1, and IL-1β in serum was up-regulated in the model group compared with the normal mice, while Dex-L and Dex-L-MVs efficiently reduced their expressions (**Supplementary Figure 11**). Thus, macrophage delivery of Dex-L can potentially reduce the inflammatory cues and balance the immune environments *in vivo*.

DISCUSSION

Idiopathic pulmonary fibrosis is a chronic and progressive lung disorder, for which only two drugs, nintedanib (NDN) and pirfenidone (PFD) were clinically approved. Because of their strong side effect, there is an urgent need to develop safer therapeutic approaches to suppress the progression of IPF. So far, only few designed targeted drug delivery systems using particles for IPF are reported, all attempts in targeted drug delivery have relayed on the specific recognition to the surface receptor. For example, Chang employed matrix metalloproteinase-2 (MMP-2) responsive peptide (peptide E5)-modified engineered liposomes loaded with nintedanib (NIN) and colchicine (COL) that can firstly target endogenous monocyte-derived multipotent cells (MOMCs) and then be selectively delivered into IPF lungs (26). Liposomal quercetin could attenuate the bleomycin-induced pulmonary fibrosis *in vivo* by the suppression of inflammatory cytokines (27). These delivery system have their own limitations, including safety issues of inhaled nanoparticles or lower drug loading efficacy (28, 29).

Macrophages or monocytes can respond to chemotactic cues and migrate to inflammatory sites, making them a potentially attractive drug delivery vehicle. The macrophages have used their powerful phagocytic function to deliver drugs to the hypoxic region of tumors in the form of "Trojan horses" (30–32). During the development of pulmonary fibrosis, inflammation is also closely related (12, 33, 34), but as far as we know, there are no reports of fibrotic diseases that use macrophages as a potential drug delivery vehicle. M1 and M2 macrophages are distinct cell subtypes and are both involved in the pathogenesis of pulmonary fibrosis. Elective treatment in clinic for IPF remains a challenge due to low drug accumulation in lungs and imbalanced polarization of pro/anti-inflammatory macrophages (M1/M2 macrophages) (13, 26). Therefore, strategies aimed at modulation of lung macrophage phenotypes may have great potential for prevention and treatment of pulmonary fibrosis in clinical settings (5, 6, 9, 26).

CONCLUSION

Here, we developed a macrophage-based delivery system loading dexamethasone liposomes to attenuate pulmonary fibrosis *in vitro* and *in vivo*. With the help of macrophage membranes or membrane-like membranes to specifically intervene in the immune response via the specific binding on the cell membrane (35, 36), we can targeted delivery drugs into the pulmonary sites in the BLM induced fibrosis mice. In addition, to answer the question about the regulatory effect of the drug contained in macrophages on the polarization of macrophages, we also demonstrated the interactions between macrophage polarizations and drug-liposomes, further evaluated how the delivered drugs affect immune microenvironment, in particular, the macrophages infiltrations. Dex-L can inhibit the activation of CD206-positive macrophages in lung tissue and *in vitro* experiments. Similar to M2 macrophage polarization, interestingly, Dex-L was observed to exhibit an inhibitory activity on the polarization of M1 macrophages by decreased iNOS and IL-6 levels *in vitro*. We anticipate that this cell-based drug delivery strategy will speak new *in vitro*, *in vivo* in the field of endogenous immune targeting IPF therapy.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Tianjin International Joint Academy of Biotechnology and Medicine.

AUTHOR CONTRIBUTIONS

XS performed experiments, analyzed data, and wrote the manuscript. YW analyzed the data. JY designed of the study, reviewed data, and contributed to the project conception and manuscript revision. YZ, GF, and FT provided guidance and partial funding support. ZX revised the manuscript. All authors reviewed, revised, and approved the manuscript for submission.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Patient Reported Outcomes in Chronic Inflammatory Diseases: Current State, Limitations and Perspectives

Florian Tran[1,2], Jan Henrik Schirmer[2], Ilka Ratjen[3], Wolfgang Lieb[3], Philip Helliwell[4], Johan Burisch[5], Juliane Schulz[2,6], Florian Schrinner[1], Charlot Jaeckel[1], Ulf Müller-Ladner[7], Stefan Schreiber[2] and Bimba F. Hoyer[2]*

[1] Institute of Clinical Molecular Biology, Christian-Albrechts-University Kiel, Kiel, Germany, [2] Department of Internal Medicine I, University Medical Center Schleswig-Holstein, Kiel, Germany, [3] Institute of Epidemiology and Biobank PopGen, Christian-Albrechts-University of Kiel, Kiel, Germany, [4] UK and Leeds Musculoskeletal Biomedical Research Unit, Leeds Teaching Hospitals NHS Trust, Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, United Kingdom, [5] Gastrounit, Medical Section, Hvidovre University Hospital, Hvidovre, Denmark, [6] Department of Oral and Maxillofacial Surgery, University Hospital Schleswig-Holstein, Kiel, Germany, [7] Department of Rheumatology and Clinical Immunology, Justus-Liebig-University Giessen, Kerckhoff-Klinik GmbH, Giessen, Germany

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Edited by:
Manuela Mengozzi,
Brighton and Sussex Medical School, United Kingdom

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Aditya A. Joshi,
National Institutes of Health (NIH), United States

***Correspondence:**
Florian Tran
f.tran@ikmb.uni-kiel.de

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Chronic inflammatory diseases (CID) are emerging disorders which do not only affect specific organs with respective clinical symptoms but can also affect various aspects of life, such as emotional distress, anxiety, fatigue and quality of life. These facets of chronic disease are often not recognized in the therapy of CID patients. Furthermore, the symptoms and patient-reported outcomes often do not correlate well with the actual inflammatory burden. The discrepancy between patient-reported symptoms and objectively assessed disease activity can indeed be instructive for the treating physician to draw an integrative picture of an individual's disease course. This poses a challenge for the design of novel, more comprehensive disease assessments. In this mini-review, we report on the currently available patient-reported outcomes, the unmet needs in the field of chronic inflammatory diseases and the challenges of addressing these.

Keywords: patient reported outcome (PRO), CID, IBD, precision medicine, rheumatology, mobile devices

INTRODUCTION - LIMITATIONS OF THE CURRENT DISEASE ACTIVITY MEASURES

Chronic inflammatory diseases (CID) such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and psoriasis are chronic inflammatory disorders at various interfaces of the human body, which, however, do not only lead to organ-specific manifestations, but indeed show strong overlaps of sites of inflammation, including the corresponding clinical symptoms. For all specific diseases, disease activity scores have been developed (e.g., SLEDAI for systemic lupus erythematodes (SLE), PASI for psoriasis). Each of these indices aim to capture organic inflammatory burden but in

a relevant proportion of patients they do not reliably reflect disease activity quantified by imaging methods or invasive diagnostics, such as endoscopy in IBD. In rheumatoid arthritis (RA), even in clinical remission or low disease activity (as defined by DAS28) about 1/3 of patients is reported to have ongoing synovitis on histological and molecular level (1). The fact, that most of these indices are complex composite scores, including laboratory values, patient's self-assessment, symptoms, and objective measures (such as the CDAI for Crohn's disease) emphasizes the difficulties in thoroughly capturing the complexity of disease manifestation. In particular, subclinical inflammatory burden in beginning flares or insufficient disease control is hardly detectable (or distinguishable from unspecific symptoms with non-inflammatory etiology), raising the need for adequate methods for screening of these seemingly hidden disease processes. Thus, also the diagnosis of CID can be challenging if the symptoms are overseen as exemplified by the underdiagnosis of psoriasis-arthritis (PsA) by about 15% in patients with psoriasis (2), even though this population of risk (with a lifetime incidence of 6-42% of developing PsA) is easy to identify since skin manifestations precede joint disease in most of the cases.

As CID are incurable diseases and typically have an onset in young or mid-aged adults, the patients and providers need to find individual therapeutic and monitoring strategies for this problem for many decades. Unresolved chronic inflammation leads to chronic destruction of affected tissues, such as synovial tissue, cartilage and bone in RA. The current disease activity scores elude to describe or quantify the grade of irreversible tissue destruction, which accumulates over time if the inflammation is not properly controlled and accounts for long-term impairments of physical and social function. The Patient's and Physician's Global Assessment (PaGA/PhGA) can give a hint toward possible impairments with a higher sensitivity (3) and are thus integrated in a few scores (such as the CDAI for RA, Mayo score for ulcerative colitis), but still do not capture all possible symptoms (like psychological co-morbidities) or reflect the specific problems in the necessary granularity.

Beyond the somatic disease activity, CID also affect various aspects of life, e.g. quality of life, fatigue or social functioning and should thus be taken into account in treatment decisions by physicians (4). Of note, significant patient-physician discordance of disease activity is a frequently reported phenomenon (5, 6). This discrepancy may significantly reduce the likelihood of reaching remission in composite-scores used for measurement of disease activity, which complicates the application of treat-to-target approaches (7). Major non-inflammatory factors contributing to such discordance and, thus, complicating the interpretation of disease activity measures and reducing the likelihood of remission defined by composite scores are co-morbidities like fatigue, chronified pain-syndromes, anxiety and depression (7–9).

Therefore, in addition to objective measures (such as laboratory results) as well as the PhGA and PaGA, objectified and specified self-evaluations of disease, co-morbidities and social impairments are important to improve medical care.

PROS: HOW CAN THE PATIENT'S VIEW HELP US IN ASSESSING THEIR DISEASES?

Addressing this lack of structured assessment, patient-reported outcomes (PROs) are standardized and validated instruments that generate numerical data representing the patients' perception and view on the burden of a disease and its treatment (e.g., symptoms, disease course, treatment effects) (10–13). PROs have been developed in research settings but are increasingly used in clinical practice and considered an essential part of comprehensive patient assessment. Additionally, shared decision making is an important part of modern therapeutic strategies (14, 15).

Longitudinal use of PROs may help tracking the patient's perspective (e.g. regarding quality of life, disease activity, functional capacity, psychological health) over the course of the disease or in response to treatment modifications (10, 13). In shared decision making, PROs are one pillar of therapy guidance.

Conceptually, PRO instruments can be categorized into generic or disease-specific measures (10). Generic measures do not target a specific disease type but can be applied across different diseases and thus, allow cross-disease comparisons. Generic PROs assess, for example, overall quality of life (established and often used instruments are e.g., EuroQoL, SF-12 and SF-36) and focus on general aspects like self-care, mobility, and physical and mental function (10). Classically, PROs can be subdivided into different domains addressing different areas of life and disease symptoms i.e. fatigue, pain, depressive symptoms, movement disabilities, which are probably the biggest domains that need to be covered.

However, and different to classical objective measures of disease activity, PROs might be influenced by many other factors, as e.g., by other co-existing diseases, psychological disorders that are not related to the disease of interest, social or financial problems (16).

Disease-specific PRO measures are constructed for a specific patient population, a specific disease, functions or symptoms (10). A number of disease-specific PROs have been developed for different chronic inflammatory disease conditions, including IBD (17) and rheumatic disease conditions (18). Most of the questions in these disease-specific PROs target a respective organ system and related symptoms. In IBD, for instance, bowel movements, bloody stool and abdominal pain are obvious questions, and PROs for rheumatoid arthritis center around functional capacity, and pain.

PROS IN RHEUMATOLOGY

In rheumatology, international consortia have been formed to foster the development of PROs. OMERACT ("Outcome Measures in Rheumatology") is such an initiative (19) which recommends measures that meet certain predefined criteria (e.g.

truth, discrimination and feasibility) (19). Some commonly used PROs in rheumatology are described below.

Many PROs contain one question related to the overall disease activity or the overall health, which can be rated by the patient on a Visual Analogue Scale (as a PaGA) (20). PaGA correlates moderately with more objective measures of disease activity, but is also influenced by non-rheumatic factors, such as education or the cultural background of a patient (5, 20). Because PaGA mostly focus on the disease activity in the form of symptoms and pain at the main organ side (e.g. the joints), they rarely sufficiently assess the systemic disease process which includes systemic inflammation driving somatic (e.g. vascular and metabolic disease) as well as psychological impairments. PaGA is incorporated in classical disease scores, such as Disease Activity Score with 28-joint count (DAS28) or Simplified Disease Activity Index (SDAI), both used in rheumatoid arthritis, and is, therefore, already part of a more comprehensive assessment approach (21).

High rates of anxiety and depression (about 10 to 40%) have been reported in RA and PsA (9, 22) using standardized screening instruments such as Patient Health Questionnaire (PHQ)-9 or Hospital Anxiety and Depression Scale (HADS) which have overall well diagnostic performance in rheumatic joint diseases (23). Many patients in remission as determined by DAS28 have persisting pain, pointing on one hand toward the pathophysiology of chronic joint pain in rheumatic diseases and on the other hand toward the insufficiency of end-point definitions without PROs (24). Classical PROs used in rheumatology are scores such as HAQ-DI, which focusses on activities of daily life. Other PROs used in rheumatoid arthritis (RA) are the Routine Assessment of Patient Index Data-3 (RAPID-3; covering pain, PaGA and functional impairment) and the Rheumatoid Arthritis Impact of Disease (RAID) instrument (covering pain, functional disability, fatigue, emotional well-being, sleep, coping and physical well-being) (25, 26).

The Psoriatic Arthritis Impact of Disease (PsAID) questionnaire is an instrument to measure impact of the disease (PsA) on different domains and dimensions of the patient's health (27), including pain, fatigue, skin problems, social participation, and work and/or leisure activities (27). Further questionnaires, like the Toronto Psoriatic Arthritis Screening (ToPAS) tool (28), the Psoriasis Epidemiology Screening Tool (PEST) (29), the Psoriatic Arthritis Screening and Evaluation (PASE) (30) and the Early Psoriatic Arthritis Screening Questionnaire (EARP) (31), have been established to target the need for early detection/screening of disease processes, and their relevance have been independently validated (2, 32). PROs have been incorporated in recent therapy goal definitions and activity scores, exemplified by the Minimal Disease Activity (MDA, containing the HAQ) (33) and the PASDAS (including SF-36 questionnaire) (34).

PROS IN INFLAMMATORY BOWEL DISEASE

Similar to other chronic inflammatory disease conditions, IBD is characterized by relevant perception gaps between providers and patients, both for intestinal symptoms and social or functional impact of disease (35–37). The available most comprehensive measures for disease activity are the Crohn's Disease Activity Index (CDAI) for Crohn's disease and the Mayo score for ulcerative colitis. The CDAI, for instance, is a complex composite score but does not reflect impact of disease in the patient's daily life (38). The patient reported 2-item (PRO2) and 3-item (PRO3) are sub-scores of the CDAI, that cover stool frequency, the presence of abdominal pain and include the patient's general well-being (PRO3) (39) and are currently increasingly used in clinical trials. However, many available PROs might correlate well with other composite disease scores (e.g. PRO2/3 correlates well with CDAI), but do not with objective disease activity markers, such as endoscopic scores or stool biomarkers of inflammation (40–42). Therefore, an important goal is to develop PROs that correlate better and more consistently with endoscopy-defined disease activity. However, even improved PROs will not completely bridge the discrepancies of symptoms and endoscopic disease activity and thus need to be regarded as important cornerstones but not the exclusive therapy guidance parameters.

In another approach to create a more comprehensive PRO which covers both perceived disease activity and classical patient-reported functionality, a simple, rapid tool to measure disease control from the patient's perspective was developed and validated in 2013 - the IBD-control questionnaire (43). This questionnaire comprises 13 items with the four core domains physical, social, and emotional functioning, and treatment as well as a VAS (43). Other disease-specific PROs to measures e.g. disease-specific quality of life (IBDQ) (44), fatigue (IBD-F) (45) and disability (IBD disability index) (46, 47) in patients with IBD are also available. The IBDQ considers intestinal symptoms, systemic symptoms, social aspects, and emotional aspects (44) and the IBD disability index covers body function, body structures, activities and participation, and environmental factors (46, 47). Also a range of generic PROs are commonly applied in IBD patients including instruments that measure depression and anxiety (BDI, HADS, PHQ-9) (48–53), and sleep quality (PSQI) (54, 55). These PROs have also been acknowledged as useful measures, complementary to and correlating with the CDAI or Mayo score, to produce a comprehensive disease assessment in clinical trial and real life settings (56, 57). In IBD patients, major depression is present in ~9% and major anxiety in ~18% (52). The PHQ-9 had the highest sensitivity (95%) in detecting depression and suicide ideation in a validation study among other available PROs and thus can be used as a good screening tool for depression (58), as these co-manifestations of IBD are definitely undertreated (59).

In different European countries, such as Denmark, the Netherlands and the UK, as well as in the US, e-Health tools for the monitoring of IBD have been developed, with some of them being directly linked to the health care system (60). With the use of such e-Health applications, patients can receive treatment recommendations online and the treating physician can decide – upon review of the results of the PRO that has been completed online – whether it is required to see the patient in

person in the clinic. On a parallel note, a doctor's appointment might also be unnecessary if the results of the online PRO indicate that the patient's disease is currently inactive.

PROS AND COMORBIDITIES

Besides the direct disease-related inflammatory burden and symptoms, other co-morbidities (either prognostically complicating disorders or diseases as consequences of long-lasting CID or independent co-morbidities) need to be more involved into the patient's assessment as they are associated with poorer patient-reported functional status in CID. In multimorbid patients with RA the proportion of care by rheumatology specialists is reduced (61), and thus the incorporation of the treatment of several co-morbidities is increasingly reflected in multidisciplinary treatment recommendations (62). The age-adjusted Charlson Comorbidity Index (CCI(A)) is a possible tool to assess co-morbidities (63) and has been used in oncology (64) and COVID-19 (65, 66) to predict long-term outcomes. The HAQ/HAQ-DI includes physical impairments which is an established link between perception of pain, cardiovascular and mental health (67, 68), delivering a more comprehensive picture of the individual's everyday life, while distinguishing disability due to disease activity from co-morbidity can be difficult in CID. Thus, better tools to assess the individual role of co-morbidities need to be developed.

The association of the organic comorbid "collateral damages" of chronic inflammation and neuropsychiatric dysfunctions can be mechanistically linked in an immunological manner (69). Inflammatory cytokines can lead to persistent changes in CNS immunity, subsequently facilitating alterations in CNS function and thus i.e. skew emotional states toward depression (70). In parallel, chronic systemic inflammation leads to metabolic changes favoring accelerated atherosclerosis (71, 72) and dyslipidemia (73).

ARE PROS COMMONLY USED IN CLINICAL PRACTICE?

PROs are important tools to monitor and document the patient's health state in clinical trial settings to compare outcomes between treatment groups, without information on individual patient's results. In clinical practice, PROs could be used as accurate and quantitative measures of the individual patient's needs, providing an extra layer of information besides the clinical assessment to guide the long-term therapy (74). Despite the potential of PROs to improve healthcare in CID and to foster shared decision-making, they have not been broadly implemented in the clinical routine. To overcome this, pioneering efforts promoted the increasing use of PROs in the clinical routine in certain regions, such as Denmark, by using eHealth applications (60). However, some PRO measures are relatively comprehensive (covering multiple different domains) and require a significant time effort by the patient to fill them out. Therefore, shorter but reliable tools should be developed to increase the response rate and to decrease the time and effort required to complete them (13). Based on this approach, validated short forms of several questionnaires have been developed, e.g. the short IBDQ (SIBDQ) and PROMIS short forms with 5-10 items, to increase the feasibility of multiple assessments in longitudinal trials and clinical practice. The main limitations, however, are shifts in the response pattern to PROs, which might develop over time in an individual patient due to conditioning to the questionnaires and coping with own symptoms (response-shift bias) (75).

The implementation of web-based assessments like electronic questionnaires represents another way to further promote the use of PROs and to save time and resources (10, 13). Simple compound scores could be used more often and could thus play a role as part of online tracking tools for patients. By monitoring their disease activity online with a simple scoring system, added up by increasingly available point-of-care-tests (POCT), such as fecal calprotectin, can help to evaluate disease activity in a setting like the current COVID-19 pandemic. Furthermore, information from PROs can be used as a trigger to initiate further examinations, e.g. additional laboratory analyses. For example, fatigue correlates with inflammatory activity and iron deficiency in IBD patients (76). Thus, if IBD patients report fatigue, this might guide the treating physician toward further iron tests or more comprehensive assessment of disease activity.

The structured collection of longitudinal and cross-sectional data might also contribute to identifying (novel) PROs for disease prediction. Patient-reported scores have revealed to be predictive of flares of specific diseases [e.g. multiple sclerosis (77)] and, indeed, flare specific questionnaires have been developed for some diseases (78). The most important point, however, is that PROs allow us, to some extent, to identify and address the disparity between the physician's global assessment that is far more attached to objective measures of inflammation and the patient's perception of disease activity. In a setting, where shared decision making is the norm, this will help us to set common ground and to define common goals beyond the pure clinical definition of remission.

WHAT ARE THE FUTURE NEEDS FOR PROS?

Defining multidimensional measures for disease activity and co-morbidities based on PROs, physician assessment, imaging studies and molecular markers remains a challenge for the future management of chronic inflammatory diseases (79). The optimal PRO needs to either capture the inflammatory burden (even if subclinical), disease-related symptoms/co-morbidities or challenging disabilities in everyday life or identify patients at risk for disease progress. Examples of such evolving PROs are the IBD-Control and the RAID instrument.

More systematically use of PROs might be promoted by the technical advances and digitalization efforts in healthcare and

thus increase the test populations for better validation of tests (80). Widespread use of waiting room devices, patient "disease activity apps" and further development and use of disease-specific POCT ("inflammometers") could improve CID healthcare.

The further development of wearable devices, which can track vital signs, motion, stress and sleeping behavior in a real-time fashion give rise to the question, whether this considerable amount of patient data can be integrated in "Next Generation PROs". These wearables are subjects of ongoing trials in patients with neurodegenerative disorders (81). A particular example is the assessment of fatigue, which correlates well with disease activity. In-depth assessment *via* questionnaires could be related to different motion parameters to identify potential device-derived disease activity measures such as reduced daily exercise, and first trials already hint toward the benefits of device-driven therapy guidance (82).

Combining these patient-centered measures with provider's assessments (including laboratory measures, imaging methods) to an integrative disease activity profile might be the key for precision medicine in CID care.

CONCLUSIONS

PROs are important for clinical management and research, as they represent a cornerstone of more personalized approaches in medicine. However, depending on the specific disease entity, the available PROs only partially reflect actual disease activity as assessed by more objective criteria like endoscopic scores. The development and usage of PROs capturing disease activity more precise for individual therapy guidance is crucial and thus they need to be implemented more widely in clinical routine.

AUTHOR CONTRIBUTIONS

FT, IR, WL and BH conceptualized and drafted the initial manuscript. All authors reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Imaging Inflammation – From Whole Body Imaging to Cellular Resolution

Tuula Peñate Medina[1], Jan Philip Kolb[2], Gereon Hüttmann[2,3], Robert Huber[2], Oula Peñate Medina[1,4], Linh Ha[5], Patricia Ulloa[6], Naomi Larsen[6], Arianna Ferrari[1], Magdalena Rafecas[7], Mark Ellrichmann[8], Mariya S. Pravdivtseva[1,6], Mariia Anikeeva[1], Jana Humbert[1,6], Marcus Both[6], Jennifer E. Hundt[9] and Jan-Bernd Hövener[1]**

[1] Section Biomedical Imaging, Molecular Imaging North Competence Center (MOIN CC), Department of Radiology and Neuroradiology, University Medical Center, Schleswig-Holstein Kiel University, Kiel, Germany, [2] Institute of Biomedical Optics, University of Lübeck, Lübeck, Germany, [3] Airway Research Center North (ARCN), Member of the German Center of Lung Research (DZL), Gießen, Germany, [4] Institute for Experimental Cancer Research (IET), University of Kiel, Kiel, Germany, [5] Department of Dermatology, Allergology and Venereology, University Hospital Schleswig-Holstein Lübeck (UKSH), Lübeck, Germany, [6] Department of Radiology and Neuroradiology, University Medical Centers Schleswig-Holstein, Campus Kiel, Kiel, Germany, [7] Institute of Medical Engineering (IMT), University of Lübeck, Lübeck, Germany, [8] Interdisciplinary Endoscopy, Medical Department1, University Hospital Schleswig-Holstein, Kiel, Germany, [9] Lübeck Institute for Experimental Dermatology (LIED), University of Lübeck, Lübeck, Germany*

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Edited by:
Oliver Distler,
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Reviewed by:
Yang Xiao,
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Osaka University, Japan

***Correspondence:**
Tuula Peñate Medina
tuula.penate@rad.uni-kiel.de
Jan-Bernd Hövener
Jan.hoevener@rad.uni-kiel.de

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Imaging techniques have evolved impressively lately, allowing whole new concepts like multimodal imaging, personal medicine, theranostic therapies, and molecular imaging to increase general awareness of possiblities of imaging to medicine field. Here, we have collected the selected (3D) imaging modalities and evaluated the recent findings on preclinical and clinical inflammation imaging. The focus has been on the feasibility of imaging to aid in inflammation precision medicine, and the key challenges and opportunities of the imaging modalities are presented. Some examples of the current usage in clinics/close to clinics have been brought out as an example. This review evaluates the future prospects of the imaging technologies for clinical applications in precision medicine from the pre-clinical development point of view.

Keywords: MRI, PET, SPECT, optical imaging, Optical coherence tomography (OCT), precision medicine, Two-Photon microscopy (TPM), hyperpolarization

Abbreviations: ASL, Arterial Spin Labelling; BBB, Blood-Brain Barrier; BB, MRI Black-Blood MR imaging; CA, Contrast-Agent; CBF, Cerebral Blood Flow; CBV, Cerebral Blood Volume; CD, Crohn's Disease; CLE, Confocal Laser Endomicroscopy; CNS, Central Nervous System; CT, Computed Tomography; DCE-MRI, Dynamic Contrast-Enhanced; DKI, Diffusion Kurtosis Imaging; DSA, Digital Subtraction Angiography; DSC-MRI, Dynamic Susceptibility Contrast; DSS, dextran sodium sulfate; DWI, Diffusion- Imaging; EUS, Endoscopic Ultrasound; FDG, Flurodeoxyglucose; FSE, Fast Spin-Echo; GFP, Green Fluorescent Protein; GI, Gastrointestinal; H&E, haematoxylin and eosin; IBD, Inflammatory Bowel Diseases; ICG, Cardiogreen; MaRIA score, Magnetic Resonance Index of Activity; MI, myocardial infarction; MMP, Matrix Metalloproteinase; MRI, Magnetic Resonance Imaging; MTT, Mean Transit Time; NBI, Narrow-Band Imaging; NIR, Near-Infrared; OCT, Optical Coherence Tomography; PACNS, Primary Angiitis of the CNS; PAI, Photoacoustic Imaging; PALM, Photo-Activated Localization Microscopy; PCASL, pseudo-continuous ASL; PET, Positron Emission Tomography; RFP, Red Fluorescent Proteins; SNR, Signal to Noise Ratio; SPECT, Single Emission Computer Tomography; SSFP, GE Steady-State Free Precession Gradient Echo; STED, Stimulated Emission Depletion; TB, Total-Body; 3D T1 FSE 3-dimensional, T1-weighted fast spin-echo sequence; TNF, Tumor Necrosis Factor; TPM, Two-Photon Microscope; UC, Ulcerative Colitis; UCEIS, Ulcerative Colitis Endoscopic Index of Severity; US, ultrasound; VDO, Vedolizumab; VWI, Vessel Wall Imaging.

INTRODUCTION

The frequency selective perception of electromagnetic waves is certainly one of the most astonishing achievements of evolution. The benefits of *seeing* were so striking that almost all species have picked up the concept in one way or another.

In medicine, visual inspection has always been the first line of assessing health and disease. As civilization advanced, so have the methods that help us *see*. Today, modern imaging methods allow us to visualize microbes, soft tissue, motion, specific antibodies, brain function, metabolism, and much more. An unprecedented plethora of imaging methods is available, not only to diagnose a patient, but to understand the mechanisms of life and disease.

New methods are being added to the quiver continuously, and hitherto inaccessible information becomes available. Different methods capture different aspects, and their combination adds up to a more complete picture of reality.

As treatment options explode, treatment control and choosing the right treatment for the patient becomes ever more important. Here, imaging is a key component to make *personalized medicine* come true: treating each patient effectively, efficiently, and individually.

Modern imaging methods, however, are just as complex as life and disease. Dedicated research communities have formed to face this challenge. In this review, we focus on the advances in imaging inflammation. It summarizes the results of the International Symposium (PMI 2020 Inflammation Medicine From Bench to Bedside) arranged by the German Excellence Cluster Precision Medicine in Chronic Inflammation (PMI) in Hamburg on 2020. For each methods, we provide a brief introduction into the technology and describe applications with respect to inflammation.

MRI

Magnetic Resonance Imaging (MRI) is the gold standard when it comes to 3D, tomographic soft tissue, and functional imaging. Without ionizing radiation and only few contraindications it has become the method of choice for many diagnostic needs. Applications include imaging anatomy (1), flow (2), brain activity (3), microstructure (4), and, to some extent, metabolism (5) – all non-invasively and *in vivo*. To do so, MRI is taking advantage of the fact that nature has provided for small, magnetic sensors that are abundant in biological tissue – the magnetic moments of nuclear spins. The strongest magnetic moment of a stable atom is that of hydrogen ^{1}H – the most abundant element in our body (approx. 10^{25} – more than all stars in the known universe). These magnetic moments can be excited by electromagnetic waves and emit a similar signal in return. These intrinsic sensors probe their surroundings and convey unique information that allows distinguishing tissues or molecules e.g. gray brain matter from the white one, or choline from creatine. From these data, the images (or spectra) are reconstructed.

Modern MRI systems can be programmed in many ways to yield images weighted by selected properties. Common examples include rather physical parameters such as T_1, T_2 or susceptibility weighting, physiological parameters such as perfusion, or structural parameters such as diffusion. While there is no dedicated "inflammation weighting", some parameters were established as surrogate markers for inflammation; these will be discussed in the following.

While these images have shown great value for diagnostics, it should be kept in mind that they are not photographs but maps of abstract quantum mechanical or physical parameters. In the following, we review selected aspects of MRI with respect to imaging inflammation of vessels and the gut. In addition, we discuss the application of hyperpolarized MRI, which allows metabolic imaging in real-time.

Imaging Perfusion in Inflammation With MRI

Background

The term perfusion refers to the transportation of oxygen and nutrients from the blood to tissues and organs by means of capillaries. In several brain diseases and pathologies, the blood supply is altered, which influences the perfusion of the affected areas. Therefore, the quantification of tissue perfusion provides valuable information to assess clinical diagnosis and medical treatment (6). Magnetic Resonance Imaging (MRI) can be used to measure perfusion levels without the use of ionizing radiation. Using this method, perfusion maps are calculated, providing a visual tool to support the clinical diagnosis of inflammatory brain diseases. By using MRI, the hemodynamics of perfusion can be described by means of various parameters, such as Cerebral Blood Flow (CBF) and Volume (CBV). Additionally, perfusion can also be characterized by the average time required for a particle (e.g., blood cell) to move through the vasculature (Mean Transit Time - MTT) and the particle velocity (7).

With MRI perfusion can be measured using exogenous or endogenous tracers. The most commonly used methods are Dynamic Susceptibility Contrast (DSC-MRI) (8), Dynamic Contrast-Enhanced (DCE-MRI) (9, 10)—both relying on the injection of a gadolinium-based external contrast-agent (CA)—and Arterial Spin Labelling (ASL) (11), which uses the water molecules in the blood as an endogenous tracer.

Applications

By tracking a CA bolus through the blood vessels, DSC-MRI reflects hemodynamic information as a hypointense signal in T_2 or T_2^* weighted images due to the increase in magnetic susceptibility of the CA in the blood (12). DSC-MRI is the standard for measuring perfusion in the human brain with MRI (12, 13), like in strokes and brain tumors. Additionally, this technique can provide information that helps differentiate malignant brain lesions such as metastases, lymphoma, and microvascular leakiness (14, 15).

DCE-MRI, sometimes called Permeability MR, is the standard approach for the measurement of perfusion outside the brain (16), e.g. in the liver (17) or prostatic (18). Here, the

shortening of the relaxation time T_1 by CA results in increased signal on, T_1 weighted images, where the CA accumulates. The time course of the MR signal, reflects the response of the target tissue to the CA's arrival. Providing quantitative information on the integrity of the Blood-Brain Barrier (BBB), tumor growth factors, and response to treatment.

The BBB permeability is the main neuroinflammatory phenomenon that can be assessed with DCE-MRI (19), for example, while monitoring the active phases of multiple sclerosis. In principle, the integrity of the BBB does not define an inflammation process "per se", but most of the neuroinflammatory activity affects the integrity of the BBB (20).

In contrast, ASL uses magnetically labeled water in the blood as an endogenous CA. There are several ASL techniques—which mainly differ on the characteristics of the labeling method—with pseudo-continuous ASL (pCASL) (21) being the method of choice in the clinical routine (22). It is an entirely non-invasive technique, able to provide absolute values of blood perfusion in tissue. By using this technique, it is possible to obtain perfusion territory maps that can provide invaluable information for the treating, planning and monitoring of cerebrovascular diseases, tumour blood supply, and vessel malformation (23). Recent advances in ASL aim to identify the specific territory that is supplied by a specific artery. This territorial-ASL, also called selective-ASL, allows to determinate and visually pinpoint not only perfusion territories but also flow, providing patient-specific information for the diagnosis of cerebrovascular disease (23).

Imaging Inflamed Vessel Walls

Background

For the workup of intracranial arteriopathies, conventional angiographic methods, including Digital Subtraction Angiography (DSA), Computed Tomography (CT), and magnetic resonance imaging (MRI) are routinely employed. Still, these methods can only depict the lumen and evaluation of disease status and progression depends on the extent of change in luminal diameter. For the differential diagnosis of arteriopathies, visualization and analysis of the artery walls can provide valuable information. While direct measurement of wall thickness is not possible in clinical MRI scanners due to the limited spatial resolution, visualization of diseased thickened or contrast-enhancing vessel segments is feasible with MR Vessel Wall Imaging (VWI). To depict the arterial wall, a high contrast between the vessel lumen and the wall is needed. The signal from flowing blood can be suppressed by special MR sequences, so-called Black-Blood MR imaging (BB MRI), increasing the contrast between the vessel wall and lumen. A frequently used sequence for BB MRI is a pre-and postcontrast 3D T1-weighted fast spin-echo sequence (3D T1 FSE), which effectively suppresses the signal from flowing blood and providing full brain coverage within an adequate examination time (24, 25). The signal from flowing blood is suppressed primarily by intra-voxel signal dephasing due to the velocity distribution within the imaging voxel and the outflow of the blood from the imaging slice during examination (26, 27). To improve blood signal suppression further, the sequence can be complemented by additional flow suppression modules (28, 29).

To reduce the artificial thickening of the vessel wall due to partial volume effects, a sufficiently high submillimeter resolution is required. However, the high spatial resolution comes at the cost of a low Signal to Noise Ratio (SNR) and a longer examination time, which might lead to motion artefacts. Thus, commonly used isotropic voxel sizes range between 0.5 – 0.8 mm^3. MR imaging at higher magnetic fields (7T or more) can improve the SNR (30). The development of new acceleration techniques can reduce the examination time (31) while maintaining or increasing the spatial resolution.

Application

Vasculitis: Central Nervous System (CNS) vasculitis is categorized as either idiopathic Primary Angiitis of the CNS (PACNS), as CNS manifestation of systemic rheumatologic diseases or associated with infection. The diagnosis of PACNS is challenging since valid biomarkers are not available. PACNS can present with a wide range of nonspecific symptoms like headache, stroke/transient ischemic attack, cognitive dysfunction, and seizures. Treatment options include glucocorticoids, immunosuppressive agents as cyclophosphamide, and the anti-CD20 monoclonal antibody Rituximab. The diagnosis is mainly based on cerebrospinal fluid analysis, typical findings in MRI and DSA, and biopsy. Imaging plays an important role in the exclusion of differential diagnoses. Digital subtraction angiography can reveal typical findings (**Figure 1**, left) but is reported to have low sensitivity and specificity (32). In the recent past, MR VWI has emerged as an important supplementary tool not only for the detection of parenchymal changes but for the improved visualization of the vessel wall and vessel pathology in the differential diagnosis of CNS vasculitides. In acute vasculitis, the arterial wall appears circumferentially thickened and strongly and homogeneously contrast-enhancing (**Figure 1**, right). The pattern of distribution in the cerebral vasculature is typically multifocal and segmental. In patients presenting with stroke, VWI findings can aid in distinguishing vasculitis from other etiologies, including intracranial atherosclerosis and reversible cerebral vasoconstriction syndrome (33–38), and in monitoring the therapy response. Moreover, MR VWI can assist in identifying a target lesion if a biopsy is indicated.

Intracranial Aneurysms: Aneurysms of the intracranial arteries were reported to have a prevalence of up to 3%. They are often incidental findings on neuroimaging and generally harbor a low risk of rupture (39, 40). In the case of a subarachnoid hemorrhage following rupture of an intradural aneurysm, a devastating outcome with persistent severe neurological deficits or even death is frequent (41). Therefore, risk stratification of patients diagnosed with an unruptured intradural aneurysm is crucial, but optimal management remains controversial. Recently, wall enhancement in intracranial saccular aneurysms on MR vessel wall imaging has been associated with a higher risk for rupture (42–46) (**Figure 2**). Experimental studies indicated that flow triggered inflammation in the vessel wall (specifically, macrophage invasion in the vessel wall) may cause formation and growth of intracranial aneurysms (47–52). Moreover, recently published results found an association of inflammatory processes in the aneurysm wall with contrast enhancement on MR VWI (45, 53–56). Therefore, wall

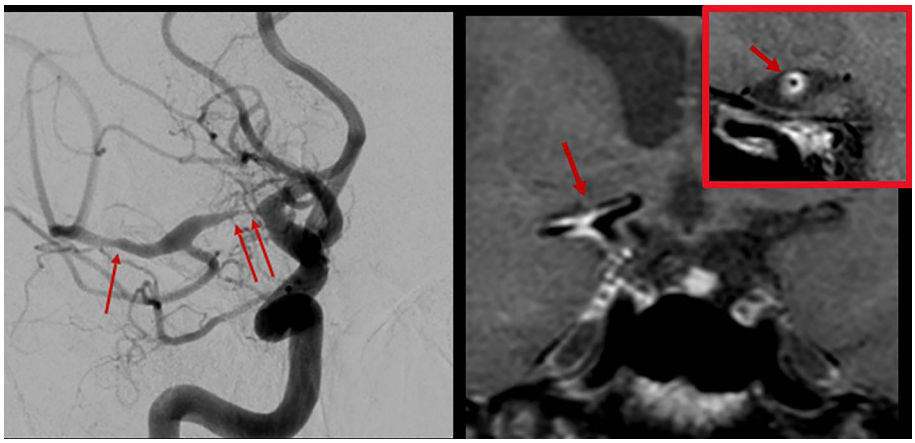

FIGURE 1 | Digital subtraction angiography of the brain with injection in the right internal carotid artery in a patient with varicella-zoster vasculitis (left). Multiple stenoses in the M1 segment of the right middle cerebral artery were found (arrows). 3T MR vessel wall imaging (right) shows strong contrast enhancement of the corresponding segments (arrows). The inset shows a transverse section through the proximal M1 segment with circumferential wall enhancement pattern.

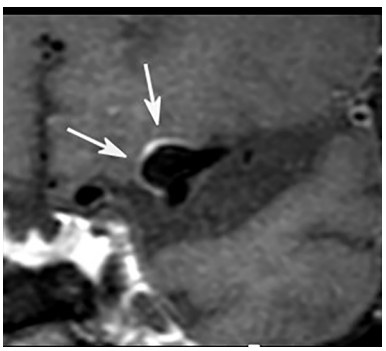

FIGURE 2 | Postcontrast 3T MR vessel wall imaging of a 5 mm aneurysm at the middle cerebral artery bifurcation. Note the strong wall enhancement (arrows) as a possible marker for visualization of wall inflammation.

enhancements may serve as a biomarker for inflammatory processes associated with wall destabilization and a higher risk for rupture of intracranial aneurysms and could aid in the risk stratification of patients with an incidental aneurysm.

Imaging of Small and Large Bowel in Patients With Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Diseases (IBD), including Ulcerative Colitis (UC) and Crohn's Disease (CD), are chronic inflammatory disorders characterized by sequences of flares with active symptomatic disease and periods of remission. While UC is typically restricted to inflammation of the mucosa and the submucosa of the large bowel, CD is a transmural process with manifestation in the gastrointestinal tract from the mouth to anus, predominantly at the terminal ileum, inducing stenoses and fistulas. IBD are disabling, life-long disorders associated with an increased risk of colorectal cancer. Typical medications include steroids, 5-aminosalicyclic acid products, immunomodulators, and biologicals like Tumor Necrosis Factor (TNF) inhibitors. Complicated disease courses require a surgical procedure (57, 58). Besides clinical and serological assessment, endoscopy and video capsule endoscopy, cross-sectional imaging, including MRI, CT, and ultrasound (US), is crucial in setting IBD as first-line techniques in diagnosis, staging, and follow-up under medical therapy (59). CT enterography and MR enterography provide comparable diagnostic performances in patients with CD (60). Nevertheless, recent studies advise preferring MR enterography because of the absence of ionizing radiation, a very high soft-tissue contrast, and a lower incidence of adverse events (61). Although MRI and US are regarded as complementary methods in CD (59), most studies revealed superior accuracy of MRI for detecting the presence, extent, and activity of small bowel CD disease compared to US (62). In general, MRI protocol comprises fastening and application of hyperosmolar oral contrast agents like mannitol prior to the examination. Typical MRI sequences are axial and coronal Fast Spin-Echo (FSE) T2W sequences with and without fat saturation, axial and coronal Steady-State Free Precession Gradient Echo (SSFP GE) sequences without fat saturation, and non-enhanced coronal T1W sequence with fat saturation followed by contrast-enhanced coronal and axial T1W sequences with fat saturation. Free-breathing Diffusion-Weighted Imaging (DWI) sequences (**Figure 3**) are optional (63). MRI findings of active CD include segmental wall-thickening and hyper-enhancement after gadolinium-based contrast media, edema, strictures, ulcerations, restricted diffusion, sacculations, enlarged local lymph nodes and hypervascular appearance of the mesentery (comb sign). Several MR scoring systems have been developed to measure disease activity, e.g., the Magnetic Resonance Index of Activity (MaRIA score) (64). Due to side effects of gadolinium-based contrast media – cerebral deposition and nephrogenic systemic fibrosis – native techniques have gained

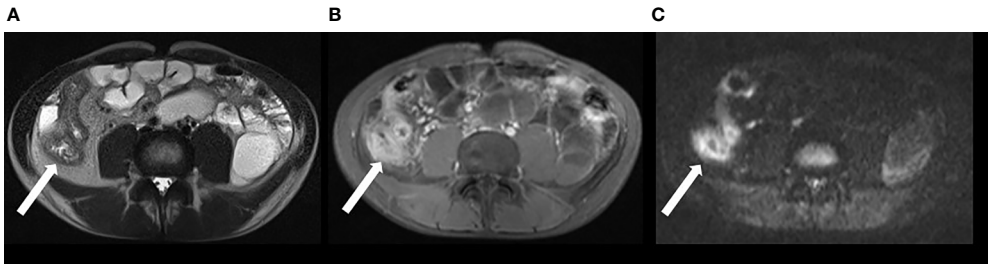

FIGURE 3 | MRI in patient with active CD involving the ileum: there is bowel wall thickening in T2w sequence **(A)** and increased contrast media uptake in T1w, fat suppressed imaging **(B)**. Inflammation is also revealed by hyperintensity in DWI **(C)**.

increasing interest in MRI (65). In recent studies, DWI was found to be mostly equal to contrast-enhanced MRI in die assessment of IBD (66, 67). There are also promising data regarding sophisticated DWI methods in the assessment of disease activity in IBD, like Diffusion Kurtosis Imaging (DKI), which reflects the heterogeneous water diffusion behaviour more accurately compared to standard DWI (68). Together with innovative tools for quantifying bowel motility (69) in patients with CD, these techniques could offer the opportunity to establish valid non-contrast-enhanced MRI protocols for patients with IBD.

Imaging Inflammation With Hyperpolarized MRI

Background

All of the MRI methods mentioned above offer unique insights into the biology and the functions of the human body. Still, many MRI applications fall short of their potential because scan times are too long or the signal to noise ratio (SNR) is too low. For example, MR is unique in being able to measure the biochemical composition of tissue non invasively and *in vivo*. This technique, MR-spectroscopy, has found important applications, but suffers from low chemical and spatial sensitivity, while the scan times are long (70–72).

At the same time, the early diagnosis of diseases before macroscopic pathologies occur is direly needed (e.g. tumors, aneurysm or chronic inflammation).

As a consequence, much research is focused on improving MRI. Most of these methods offer fractional improvements, e.g. a SNR gain of 30% by acceleration techniques. Other provide a few-fold enhancement, e.g. by increasing the magnetic field from 1.5 T to 3 T and 7 T. MRI with hyperpolarized contrast agents, however, has demonstrated to boost the signal by several orders of magnitude – e.g. 10.000 or 100.000 fold of the selected molecules. Like MRI, hyperpolarization is an inherently quantum mechanical effect. As described above, MRI is based on the magnetic moment of atomic nuclei, which is induced by nuclear spins. In some aspects, this magnetic moment behaves like the needle in a hiking compass. Like a compass, the nuclear spins align in an outer magnetic field; unlike a hiking compass, however, the spins don't all align in the same direction. Instead, the spins are distributed in parallel or antiparallel to the magnetic field following the Botzmann distribution. As spins pointing up and down cancel, only the population difference effectively contributes to MRI signal. The fraction of all spins contributing to the signals is called polarization (P):

$$P = (N\beta - N\alpha)/(N\beta + N\alpha) = \tanh(\gamma = B_0/2k_BT) \approx \gamma = B_0/k_BT$$

Where N is the occupation number of the upper and the lower energy levels α and β, $\hbar$ is the Planck constant, γ is the gyromagnetic ratio, B_0 is the magnetic field applied, k_b is the Boltzmann constant, and T is the thermodynamic temperature.

For all practical matters *in vivo*, the polarization is very, very small. In the magnetic field of the earth, ≈ 50 μT, the polarization is only a few parts in a billion – only few ppb contribute effectively to the MR signal. In a magnetic field of 1.5 T, the fraction is increased to a few in a million. In other words: 99.999% of all spins in a sample (or body) are invisible in routine MRI, leaving room for a dramatic enhancement of the MR signal and new diagnostic applications.

Several methods have been developed to increase the polarization of a solid, liquid, or gas (73–75). Usually, these methods use some spin order that is readily available in nature to increase the polarization of the target substance. For biomedical applications, hyperpolarized metabolites and gases are particularly interesting.

Applications

Biomedical MRI of hyperpolarized metabolites in solution was introduced in the early 2000s. Here a hyperpolarized CA is injected *in vivo* and several metabolites are measured (e.g. pyruvate to lactate, alanine, bicarbonate). Since then, impressive works have been published, including the first applications to humans, where real-time metabolism was detected in the brain and heart (76, 77). In the prostate, cancerous metabolism was detected before visible lesions occurred - an important step for early diagnosis and personalized medicine (76).

As for inflammation (78), it was found that the inflamed paw of an arthritis model (79) showed a higher pyruvate to lactate ratio than the control paws (**Figure 4**). The higher amount of lactate correlated to inflammation, as was validated by clinical and histological analysis. In another study the same CA (hyperpolarized pyruvate) was used to assess hepatocytes

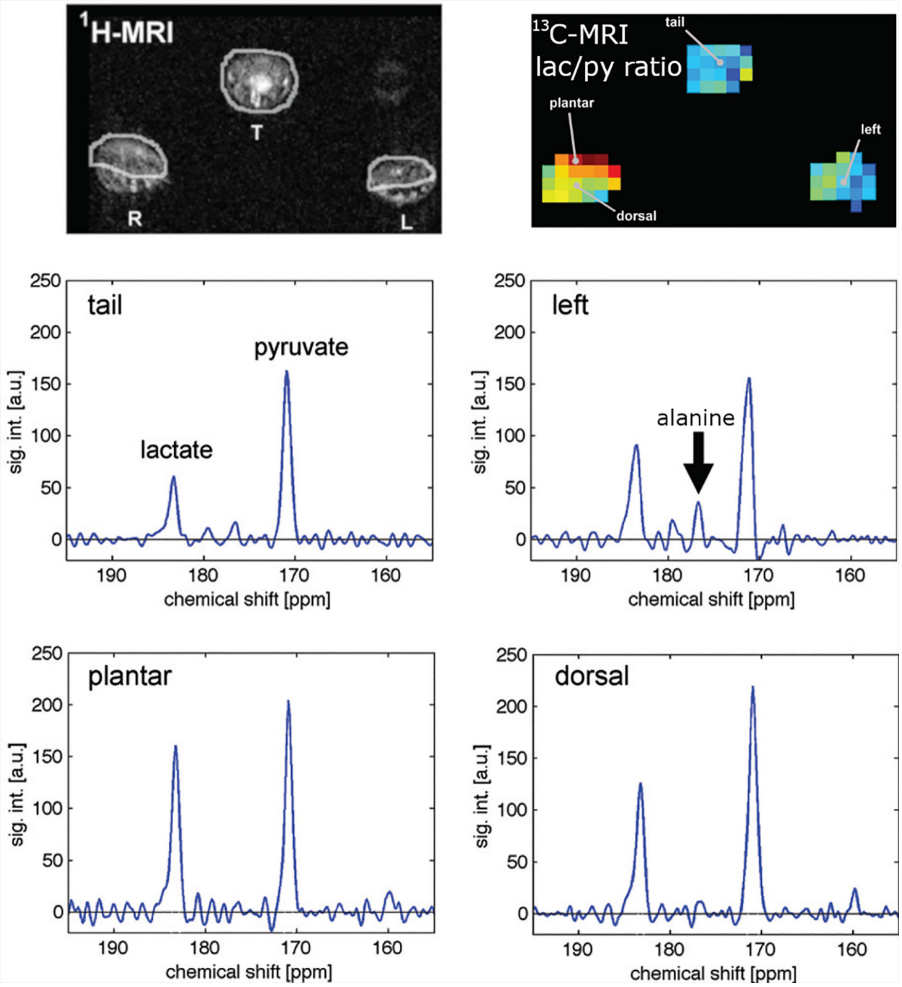

FIGURE 4 | Measuring the metabolism of an arthritis model with hyperpolarized hyperpolarized MRI: anatomical 1H MRI (top left), quantitative metabolic map of lactate-to-pyruvate ratio (top right) and corresponding 13C spectra. Arthritis was induced in the right paw of the rats while the left served as a control. Hyperpolarized pyruvate was injected and ^{13}C metabolic imaging performed. The inflamed paw exhibited a 65% increase in lactate signal and no alanine signal indicating abnormal metabolism. Figure modified from [MacKenzie et al. (79)].

necrosis in a CCl_4 rat model. The conclusions was that ^{13}C metabolic imaging with hyperpolarised [1-^{13}C] pyruvate is sensitive to inflammation (80). Lewis and co-workers showed that hyperpolarized [1-^{13}C]pyruvate can be used to evaluate the local cardiac inflammatory response due to Myocardial Infarction (MI) (81) with a broad potential across cardiovascular diseases.

Eto and co-workers (82) followed a different approach and used radicals *in vivo* for redox imaging in skeletal muscle disorders associated with inflammation.

MRI with hyperpolarized gases, Xenon-129 (83) and Helium-3 (84), provides unique diagnostic information on the human lung (85, 86). Imaging the gas distribution provides ventilation maps in 3D with high resolution (**Figure 5**), measuring the diffusion allows to assessing the lung microstructure, e.g. the alveolar condition (87). The gas exchange and function of the lung can be measured by using spectroscopic MRI, where Xenon in the airspaces can be distinguished fron Xenon dissolved in blood plasm and bound to red blood cells (88). These techniques were used to access chronic obstructive lung disease (COPD) (89), asthma (90), idiopathic pulmonary fibrosis (91) and a local inflammation (92). For example, ventilation deficits can be readily imaged with ^{129}Xe-MRI. Likewise, **Figure 5** depicts clearly the lung degradation with different pulmonary diseases obtained by ventilation ^{129}Xenon MR-imaging.

EMISSION TOMOGRAPHY: PET AND SPECT

Background

Positron Emission Tomography (PET) and Single Emission Computer Tomography (SPECT) are well-established imaging techniques in both clinical routine and pre-clinical research for a

large variety of applications. PET and SPECT rely on the administration of specific radiotracers and subsequent detection of high-energy photons. Both modalities stand out for a superb sensitivity, which translates into the detection of radioisotope concentrations in the nano to picomolar range. In the case of SPECT, the selected molecules or particles are labeled with gamma-emitting radioisotopes, whereas for PET positron-emitting radioisotopes are required. The emitted positrons are not directly detected by the scanner, but the pairs of high-energy photons that arise from the interaction between positrons and their counterparts, the electrons from the tissue. Thanks to this feature, PET offers a higher efficiency than SPECT, as the latter requires collimators to select only those photons from a certain direction. In any case, the detected photons indirectly reveal the location of the radiotracers. To extract this information, tomographic image reconstruction is required,

The radioisotopes Technetium-99m (^{99m}Tc) and Fluorine-18 (^{18}F) have remained for decades as workhorses for PET and SPECT, respectively. The latter is mainly used to label Flurodeoxyglucose (FDG); the resulting tracer ^{18}F – FDG is a commercially available glucose surrogate, and as such, it has been successfully employed to track glucose metabolism within a large variety of diseases. Additionally, a large variety of radioisotopes can be used for labeling relevant substances, from simple molecules such as water, to antibodies, drugs and even bacteria. As the radiotracers are designed to target selected biochemical processes, their distribution in time and space unveil the underlying metabolism and biokinetics. Theranostics approaches go one step beyond, so that the radiolabelled compounds, designed e.g. to irradiate malignant cells, can be also localized by means of PET or SPECT.

At present, stand-alone PET scanners have become a rarity, and bi-modal PET/CT and PET/MRI systems are used instead, not only in the clinics but also for small-animal imaging. Also, SPECT/CT scanners are commercially available; SPECT-MR still remains only restricted to rodents, although some developments aimed to bring SPECT/MR into the clinics have been reported (93). All these synergistic approaches offer both functional and anatomical information. Moreover, the information provided by additional modality helps enhance the quality of the PET or SPECT images. This, in turn, leads to improved lesion detection and, in the case of PET, more accurate quantification. In the context of imaging inflammation and infection, simultaneous PET/MRI has proved to be advantageous compared to independent scans of the two modalities (94). One concern for CT as additional modality is the increased total radiation exposure. This is obviously not the case for PET/MRI, as MRI does not require ionising radiation. In any case, latest advances in instrumentation and software have contributed to significantly reduce the delivered effective doses without jeopardizing image quality.

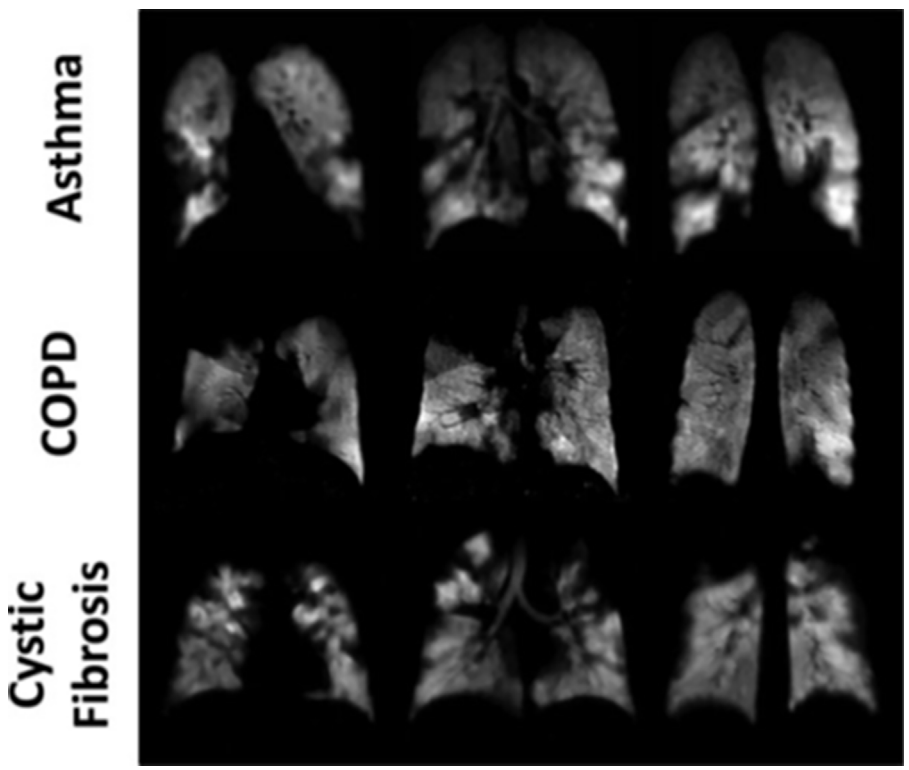

FIGURE 5 | Ventilation imaging of the diseased human lung using ^{129}Xe-MRI. Coronal ventilation images were acquired in subjects with asthma (upper row), COPD (middle row), or cystic fibrosis (lower row). Numerous ventilation defects can be seen in each of the images secondary to airflow obstruction caused by the underlying diseases. Figure taken from Mugler, J. P. et al., Journal of Magnetic Resonance Imaging (85).

Whereas visual interpretation of PET and SPECT images usually suffices for routine diagnostics, PET (and to a lesser extend also SPECT) can also provide quantitative information. Quantitative PET mainly refers to extracting from the reconstructed images the absolute amount of radiotracer accumulated in a specific region of interest within a certain time frame. This information can be expressed in terms of e.g. kBq/ml, or as a standardized uptake value and can be particularly useful to assess the response to therapy. Furthermore, the rate of tracer transportation and exchange (tracer pharmacokinetics) can be estimated from dynamic PET data in combination with kinetic modeling analysis.

Producing a PET image from the measured data is a complex process. Thanks to the increasingly growing computing power of desktop PCs and GPUs, the time required for image reconstruction has been strongly reduced. Advanced algorithms have become part of the manufacturers' software so that images are generated shortly after the scan is completed or even on-the-fly. Still, there is room for further improvements at the software level, which should go hand in hand with the corresponding advances in instrumentation to fully exploit the potential of novel components and designs (95). The recent development of Total-Body (TB) PET scanners (96, 97) is expected to boost molecular imaging and personalized medicine. Such systems allow the entire patient body to be imaged in a single scan, making a further dose reduction and faster imaging possible (e.g., 1-min scans). In particular, TB-PET opens the door to ultrahigh-resolution dynamic imaging with 100-ms short frames to capture the fast initial distribution of the radiotracer. In the last decades, organ-specific imaging devices as well as systems for intraoperative use have been developed although very few imaging concepts have reached commercial maturity. In contrast, dedicated rodent scanners, developed to provide high sensitivity and high spatial resolution, are long commercially available to support preclinical research. Current small-animal PET scanners are characterized by a spatial resolution of about 1 mm, whereas a better resolution (but worse sensitivity) can be achieved by pre-clinical SPECT systems. The progressive consolidation of zebrafish as a model organism for pre-clinical research, also to investigate inflammation (98, 99), is demanding the availability of specific PET systems and protocols (100). In this vein, some of us have started developing a dedicating system and imaging setup to allow for zebrafish PET imaging (101).

Applications

Diagnostic PET and SPECT are routinely employed in oncology, cardiology and neurosciences. Their suitability to image inflammation has been long recognized (102–106), also specifically for inflammatory bowel disease (107–115), including preclinical research on murine models (116, 117).

The specificity of PET and SPECT relies on the choice of the radiolabelled compound. Several radioactive tracers have been used for the detection of the immune system and inflammation. The gold standard of lymph node detection in surgical settings is based on the Tc-99 sulfo-colloid nanoparticle. These technetium levels are measured *via* gammascintillation counter from surgical samples or *in situ* by using gamma camera or SPECT. The sulfo-colloid meshwork role is needed to slow down the radio-ligand diffusion and ensure that the elimination of the complex will be done through the lymphatic drainage *via* lymph nodes. In this method, the technetium sulfo-colloid is injected several hours in advance of the surgical operation, and the imaging is done prior to the procedure. However, there is often no additional help to the surgeon in an intraoperative setting except the possibility to scan individual lymph nodes at the site with a radioactive detection device. An injection of optical tracer, Cardiogreen (ICG), has been used to bring this component to the surgery. The ICG injection could help to detect sentinel lymph nodes in intraoperative settings, but it can be used reliably only after skin removal and up to 1-1.5 cm deep into the tissue (118, 119). Alternatively, mini gamma cameras or freehand SPECT systems could be used. These devices have been introduced for intraoperative applications, although their use is not widespread (120).

^{18}F-FDG has been used successfully for the detection of highly active inflammation. The use of FDG is based on the fact that FDG resembles glucose enough that it is internalized by the cells that are in need of glucose. FDG cannot be further metabolized, like glucose, leading to FDG accumulation. The PET tracer ^{18}F-FDG thus allows cell imaging and cell labeling, while several different cells take it up efficiently. Macrophage labeling has been used to track the status of inflammation in arthritic patients. The macrophages are first extracted from the blood, labeled with a radioactive tracer, and reinjected into the bloodstream to follow the accumulation to the organ of interest. Several inflammatory disorders include sarcoidosis, atherosclerosis, vasculitis, IBD, rheumatoid arthritis (RA), and degenerative joint disease are imaged using immune cells. Gallium-67 (^{67}Ga) citrate, ^{99m}Tc- or ^{89}Zr-labelled leukocytes, indium-111 (^{111}In), as well as ^{18}F-FDG represent the most widely used radiopharmaceutical agents (115, 120–122). In addition to cells, bacteria have been targeted and imaged by using radiolabeled antibiotics (123). However, other preparations, like labeled murine monoclonal antigranulocyte antibodies and labeled human polyclonal nonspecific immunoglobulin G, chemotactic peptides, interleukins, chemokines, and liposomes, have been used to image inflammation (124–127). Chelates that can be coupled to different proteins, lipids, and sugars are widely used in the development of new tracers. At another level, the combination of PET with radiolabeled therapeutic agents, such as liposomal glucocorticoids, is helping to push forward drug development in the treatment of inflammatory diseases (128). It is thus to be expected that current advances in radiochemistry and radiopharmacy, together with improved imaging technology, will further contribute to consolidate PET and SPECT as indispensable tools for precision medicine.

OPTICAL IMAGING

Optics covers some of the oldest and most important forms of medical diagnosis and research. By simply looking at a patient, the shape and color perceived with the naked eye can already

provide valuable diagnostic information. The strength of optics in biomedicine is its potential for very high spatial resolution and specific contrast. Optics is capable of visualizing sub-cellular structures and stood at the beginning modern medicine. Today, optical microscopes can resolve even structures only a couple of 10 nanometers in size and using fluorescence techniques, they can provide molecular functional contrast. Since they do not use ionizing radiation or particle beams, optical microscopes exhibit very good non-destructive and even *in vivo* capabilities in contrast to other high-resolution techniques like for example electron microscopes or micro CT.

Thus, by now optical imaging and sensing is of paramount importance in clinical and medical research laboratories in form of benchtop devices. These range from standard types of reflection, transmission and fluorescence microscopes to more advanced confocal, two photon and Stimulated Emission Depletion (STED) or Photo-Activated Localization Microscopy (PALM) super resolution microscopes. But also devices like flow cytometers and cell sorters and almost all DNA sequencers use optical methods – mainly fluorescence – for sensing. Right now, the digital revolution, which enables fully electronic processing of images and photos in consumer products, is starting to have a massive impact on medical imaging (digital microscopes, camera in a pill etc.). Supported by the new possibilities offered by modern data processing units, the rise of optics in medicine will continue.

Considering *in vivo* imaging applications in a clinical setting, the eye and the skin are ideal target organs since they are very easily accessible by optical technologies. With respect to other target organs, besides the numerous biophotonic laboratory tools mentioned above to sense extracted samples or cells outside the body, the main problem of optical *in vivo* imaging for diagnosis in patients is the poor penetration of light into highly scattering tissue. Still, in many cases, it is possible to use ***endoscopes in order to deliver light to deep inside the human body***. Hence, almost all epithelial structures at "barrier interfaces" are accessible by current endoscopes. Today's endoscope technology in clinical routine almost exclusively performs simple reflection imaging, which means, simple color images of the sample are created. However, there are more advanced optical imaging techniques as mentioned above, which could provide an additional wealth of information for an earlier and more precise diagnosis of disease.

Generally speaking, it were always advances in technology which triggered a paradigm shift in the medical application of optics and opened new realms of application to use optics as tool for early diagnosis of disease, supporting treatment decisions and monitoring patient response. Recently especially with the advent of full digital imaging processing chains optical imaging is not only used to generate image but also to quantify disease stages by deriving robust parameters like layer thicknesses, vascular branching densities or tissue elasticity. This allows an observer independent quantification of health or disease status for more consistent and precise treatment decisions.

In the following, several examples spearheading the introduction of advanced optics to inflammation are described.

Fluorescent and Molecular Imaging

Background

Clear advantages for optical imaging are the fact that optical imaging devices and patient imaging are, in general, cheaper than radioactive and MRI imaging. Optical imaging is sensitive, and the theoretical resolution is excellent, allowing accurate molecular imaging. Optical imaging can also be performed more often in one person in contrast to CT or radioactive imaging, which are limited due to maximum radiation dosages. Some methods like ICG based rheumatoid arthritis imaging are in sporadic use in clinics (129). The main reason that hinders the usefulness of optical imaging in daily practice is the limited penetration depth. The optical signal can travel only a couple of centimeters at best when using Near-Infrared (NIR) wavelengths and NIR probes. This distance can theoretically be extended up to 10 cm when photoacoustic detection is used (130). Photoacoustic Imaging (PAI) combines light and ultrasound into an absorption-based non-invasive imaging technique. In PAI the ultrasound signal emerging from the thermoelastic expansion caused by optical absorption within biological tissues or the contrast agent is measured. These spatial limitations of the penetration depth are less relevant in small animal imaging, where full 3D tomography can be performed due to the small size of the animals of interest. Clinically approved fluorophores can be sensitive to their environments and give different readings based on their surroundings (131). At the moment, one brand of 3-D fluorescence optical tomography is available for a small animal. These include mice, hamsters, rats, and small rabbits. For PAI, pre-clinical instruments for small animals can be coupled with co-registered ultrasound imaging, yielding a 2D or a 3D-tomograpic image with anatomical and molecular information (132). Complicated immune reactions can be studied in disease models like Dextran Sodium Sulfate (DSS) inflicted acute and chronic IBD mouse models (133). Several ready-made NIR-fluorescent tracers that can detect enzymatic functions with great precision are commercially available. There are tracers for various immune-related targets like Matrix Metalloproteinase (MMP) recognition, angiotensin probes, and neutrophil sensing elastases (134, 135). Optical 3D imaging can be performed using the same imaging probes, which are also used in cell experiments allowing convenient molecular imaging from cells- to the tissue- and organ-imaging without extra labeling steps. While optical tracers per se are relatively small molecules - below 1000 Daltons - and can be coupled with premade linkers, they are easy to use. There are optical tracers for RNA, DNA, proteins, lipids, and carbohydrates. Although contrast agents for fluorescence imaging are optimized for maximal quantum yield some are also applicable in PAI like ICG. However, better molecular tracers are needed for useful immunological 3-D imaging. Fluorescence imaging requires always contrast agents, whereas inflammation imaging with PAI can be used to quantify the increased tissue oxygenation (136), vascularization (137), or fibrosis (138) typical for inflammation. Besides, non-targeted contrast agents like ICG, IRDye, or melanin, or targeted contrast agents like liposomes (139), microbubbles (137), or gold nanoparticles (140, 141) may

enhance or specify the photoacoustic signal. Nanoparticle imaging has been shown to offer promising results in immune cell and disease imaging studies (139, 142, 143). The excellent and encouraging results from small animal imaging should be converted to clinical applications in the future. Surgery can benefit from optical imaging with tracers and methods developed for small animal imaging. Surgeons aided by using optical cameras with fluorescent filters in surgical robots and operational microscopies with fluorescent filters allow the better gathering of the visual information on site. Optical imaging could also be used together with endoscopic imaging in gastrointestinal studies to improve IBD treatments significantly.

Applications

The human Gastrointestinal (GI) tract microbiota has been a subject of intense research throughout the 3rd Millennium. In recent years, the importance of gut microbe diversity for human health has become evident (144). Robust bacterial clusters, the enterotypes, have been described (145). They are stable bacterial communities composed of a limited number of species. Additional information about bacterial colonizing behavior and metabolism is needed to understand better the relevance of specific strains to human health and diseases like IBD. Fluorescence imaging offers a practical method to understand dynamic interactions between microbe species and microbe-host cells in the gastrointestinal tract. Optical *in vivo* imaging of either bioluminescent or fluorescent bacteria is the basis for non-invasive intestinal colonization detection. The intestine anatomy does not make the GI tract imaging simple, the irregular shape, and most importantly, the deeply embedded organ cause difficulties in 3 D- fluorescence imaging and raise special requirements for the fluorescent markers used. Transcriptional reporters have widely been used in bacterial imaging since Green Fluorescent Protein (GFP)- technology was developed (146). GFP-based imaging has proceeded *in vivo* in the mouse intestine, but the sensitivity does not meet the need to observe bacteria in the physiologically needed range (147). Bioluminescence imaging with luciferases has advantages in sensitivity compared to GFP. Notably, the lux operons are suitable for *in vivo* imaging because there is no need for added substrate, and they have been used in whole-animal imaging in the intestine (148). The background fluorescence from tissues seen in GFP-labeled bacteria can be avoided if Red Fluorescent Proteins (RFP) are used. The dual-color 3D imaging of different bacteria utilizing infrared fluorescent proteins has been presented (149), and several suitable RFPs are available at the moment. Their usage in bacterial imaging has been recently studied by Barbier and Damron (150). They compared the expression, toxicity, photo stability, spectral overlapping, and sensitivity of various fluorescent proteins in *E. coli*. The proteins likeKatushka, mKeima, and E2-crimson (151–153) with red fluorescence are the most promising candidates for the deep tissue *in vivo* applications based on their fluorescence characteristics. The protein toxicity was not a big issue, but instead, spontaneous loss of plasmid in the absence of antibiotics is evident and needs to be considered in study setups. Genetic labels are limited to the bacteria for which cloning tools are available; thus, universal fluorescent labels will offer a powerful tool for proper bacterial imaging. Universal, chemical, fluorescent stains will overcome the question of fluorescence range, while they can be used in higher wavelengths from 640-800 nm, which the fluorescent proteins will not reach. The chemical stains can be based on different chemical interactions. Recently hydrophobic membrane stains have been utilized to label *E. coli* (154). Also, electrostatic interactions can be adapted to label bacteria *in vivo* conditions (155–157). A combination of universal membrane-stain and near far-red fluorescent protein Katushka has also been used successfully with *E. coli* strains. If dual staining is used, the strains can be distinguished from each other, and their mobility can be followed (154). A similar setup could be used in the future to study the interactions of specific bacteria in the colon.

The clinical need is to support the disease diagnostics and evaluate the severity of bacterial inflammation. The most straightforward form of imaging bacteria in the clinical application is to use their endogenous fluorescence by exciting the bacteria with low-intensity violet light (405 nm) (158, 159). In several pre-clinical and clinical bacterial imaging studies, dual radioactive and fluorescent imaging has proceeded mainly using endoscopic set up (160). In these studies, the fluorescent staining was primarily done using bacterial targeting molecules like antibiotics or antibiotic peptides (161, 162), enzyme activated tracers (163, 164), or bacterial lectins (165). In clinical applications, the fluorescent markers cause extra inconvenience, while most of the dyes are not clinically approved. However, few multimodal pre-clinical studies having fluorescent markers as a second marker have been conducted (166, 167). Added fluorescent markers can be used to trace the bacteria from histological samples, differentiate the bacteria type, and evaluate the area of infection and thus aim in the future to image-guided surgery. Though the presented studies are still difficult to implement in clinics, the data collected from multimodal and more theoretical fluorescence studies will, in any case, offer new applications in bacterial diagnostics and treatments. In pre-clinical imaging, PAI has similarly been applied for the detection of different inflammatory diseases such as IBD (168–171), arthritis (172–174), and vascular inflammation (140). With the introduction of the first clinically approved photoacoustic system, the first studies now show the potential for human patient imaging (175).

Two-Photon Microscopy for Sectioning-Free Virtual Haematoxylin and Eosin (H&E) Imaging

Background

In the routine pathology workflow, single-cell layer thick sections of tissue samples required for diagnosis are created by paraffin sectioning. The method is quite a labor and time-intensive process, requiring the sample to be fixed in paraffin for about one day. It is then drained in an automatic machine, usually overnight, which means that water in the tissue is first replaced with alcohol, then with an organic solvent such as xylene, and finally with paraffin. The tissue is then poured into the paraffin and, after cooling, cut into slices of about 5μm thickness using a

microtome. These are then placed on a microscope slide. With the help of alcohol, the paraffin is washed out again and usually stained with H&E. The only current established alternative is frozen sectioning, where the sample is embedded in a medium, then flash-frozen and cut into thin slices. The reachable thickness strongly depends on the tissue but is usually a couple of µm thicker than paraffin sections. Artifacts from the freezing process or cutting are a common issue. Although frozen sectioning delivers faster results, the diagnostic quality of the sections is significantly lower than with paraffin sectioning. To establish a faster, less labor-intensive, yet high-quality alternative to thin sections, various optical imaging techniques for the creation of virtual sections have been tested in the research community and some of them were also commercialized (176–181). The tissue sample does not have to be cut, but different optical effects are used to achieve optical sectioning. In most cases, only staining is necessary as sample preparation, which results in a drastic saving of work and time. In a two-photon microscope (TPM) (182) it is exploited that fluorescence can be excited not only by one photon, which can happen anywhere in the light beam, but also by several photons of a lower wavelength that combined have enough energy to excite the fluorophore. Since these photons must be at the same place simultaneously, there is only a sufficient probability for this effect in the focus of the microscope, i.e. in a small spot. The focus can now be moved over the sample to make the dyes fluoresce point by point and create a virtual slice plane.

TPM is a standard tool in neurobiology to observe the activities of nerve cells (183). However, the setups used here usually fill an entire air-conditioned and darkened room. In addition, the titanium-sapphire (Ti : Sa) crystal lasers used are relatively maintenance-intensive, and the existing free beam paths must often be readjusted. A water cooling system is also necessary, which requires regular maintenance. There is one solution, where such a system has been engineered to be used in the clinic (184). We in our group have found that lasers with longer pulse durations in the range of a few 10 ps to a few nanoseconds (SubNs) can also be used for TPM in contrast to the usual ~200fs pulse duration (185). The same images can be obtained at constant average power if the laser's duty cycle is kept constant, i.e. longer pulses are used, and their repetition rate is reduced by a corresponding factor. The use of longer pulses has the decisive advantage that dispersion in glass fibers no longer plays a major role, and the pulses in these fibers no longer diverge, which would reduce their peak power and thus also the fluorescence signal. For this reason, the laser and the complete beam delivery system up to the microscope optics can now be constructed from glass fibers and corresponding components, which are also used in telecommunications technology. This not only makes the complete setup much more reliable but also less sensitive to temperature fluctuations and vibrations. This enables us to build the entire setup into a mobile rack that can be used anywhere and is also maintenance-free.

Applications

We use TPM of bulk tissue samples to create images that resemble standard H&E-stained slides without any sectioning and to evaluate whether it is a viable alternative. Before imaging, the bulk tissue samples are quick-stained (2-10 min) with acridine orange (nuclei stain) and sulforhodamine 101 (counterstain) to achieve an H&E compatible staining. Our home-built two-photon microscope images the unsectioned tissue samples at high three-dimensional resolution. A plane within the sample is scanned and the fluorescence from the focus is collected by two separate spectral channels to separate nuclei- and counterstain. A digital H&E-equivalent image ready for histological assessment is created from the acquired data. A porcine skin sample was successfully imaged without sectioning using our TPM microscope as seen in **Figure 6**. Compared to the preparation of H&E-stained paraffin sections of the same sample for bright-field microscopy, this took considerably less time and work. Similar image quality and features could be observed compared to paraffin sections. Other types of tissue and more samples are planned to be investigated. Moreover, we intend to further increase the speed of the TPM microscope from currently ~25 minutes/cm² up to 1-2 minutes/cm² with four times more sensitive detectors and by improving the performance of our acquisition and processing software. Also, haematoxylin and eosin (H&E) as stains will be tested to achieve a more realistic image impression. We believe that the pathology workflow can be simplified with virtual H&E imaging with TPM as an alternative to frozen- and paraffin sectioning in the future. The remaining challenges are faster imaging and data processing. It could also provide improved diagnostic accuracy by the potential combination with other imaging modalities (e.g. TPM fluorescence-lifetime-imaging) and the creation of 3D images. Further investigations will include the comparability to standard H&E staining and whether fluorescent immunostains could be used as well.

Novel Endoscopic Imaging Approaches in Inflammatory Bowel Disease

Background

Ulcerative colitis and Crohn`s disease comprise chronic inflammatory bowel diseases that cause severe damage of the integrity of the luminal gastrointestinal tract. The gold standard for the diagnosis of IBD is a combination of clinical presentation, endoscopy, and histology (186). Apart from that, endoscopy in IBD plays a major role in predicting disease severity, extent, and prognosis as mucosal healing was defined as a major therapeutic goal (187). High definition white light endoscopy (HD-WLE) is an important tool in the evaluation of IBD using various endoscopic classification score, i.e. in UC the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) (188) and the endoscopic Mayo score are the ones mainly applied in clinical routine (189). These scores focus on endoscopic findings, such as ulceration, friability of the surface, spontaneous bleeding, and mucosal edema. Since these scores are always limited to the mucosal surface, they exhibit a significant interobserver variability with sensitivities, specificities, and accuracies of 70.8– 95.3%, 67.0–100%, and 32.4–100%, respectively (190) in comparison to histological inflammation as reference (189). Though studies could demonstrate that mucosal healing, assessed after 14 weeks of treatment, correlated with long-term remission in both IBD entities, no commonly accepted definition of mucosal healing

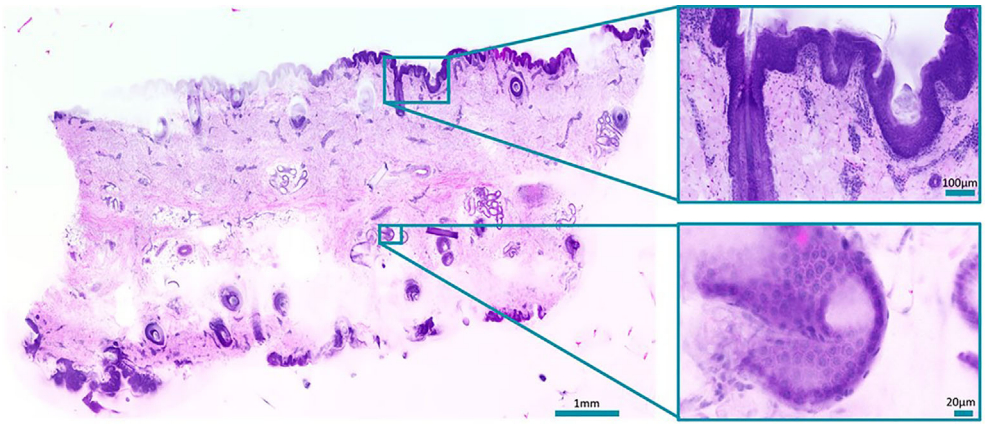

FIGURE 6 | Slide-free image of a bulk porcine skin sample stained with acridine orange and sulforhodamine 101. Zoom-ins show a hair follicle (top) and a sweat duct (bottom). Total acquisition took 13 minutes plus. 10 minutes processing time.

has been established so far. Endoscopic findings poorly correlate with histological activity and are not suitable to predict relapse in more individualized therapeutic strategies (190). Therefore, various modern imaging modalities have been explored that enhance detailed mucosa assessments in real-time, including virtual chromoendoscopy techniques, i.e. Narrow-Band Imaging (NBI), Confocal Laser Endomicroscopy (CLE), and Optical Coherence Tomography (OCT).

Digital Chromoendoscopy, Narrow-Band-Imaging

Narrow-band-imaging (NBI) utilizes optical filters to illuminate the tissue with defined wavelengths (415 and 540 nm) that are absorbed by hemoglobin but have different penetration depths. NBI, therefore, allows detailed examination of mucosal vascular and surface patterns. In assessing inflammation in IBD and predicting therapy response, divergent data have been published so far. In a prospective study by Kudo et al. 30 UC patients were longitudinally examined, showing good criteria of the Rachmilewitz score and histological markers of inflammation as well as subsequent relapse (191). In contrast, a more recent study in 64 UC patients could not predict relapse within one year of therapy (192). This discrepancy may be explained by the different scoring systems used (Rachmilewitz *vs.* Nishio score) with limitations to superficial criteria.

Endoscopic Ultrasound (EUS)

Recent data, in part unpublished, of our group evaluated the role of EUS for the differentiation of CD and UC compared to healthy controls. Combining the EUS criteria total wall thickness (TWT), mucosal/submucosal thickness, and the presence of paracolonic lymph nodes, we could differentiate between active CD and UC with 92.3% sensitivity (193). Furthermore, TWT of the recto-sigmoid colon strongly correlated to histological disease activity prior to initiation of anti-inflammatory therapy and significantly declined within the first two weeks of anti-TNF treatment preceding the changes of the superficial, endoscopic appearance by several weeks. With a sensitivity and specificity of 0.9 a cut-off value of approximately 8% reduction in TWT was calculated to predict therapy response at this very early time point (194).

Confocal Laser Endomicroscopy (CLE)

CLE enables real-time imaging of the mucosal surface with ~1000x magnification and a resolution of ~1 micron. It is based on the tissue fluorescence of the target area activated by probe emitting laser light and collecting the emitted fluorescent light at the same time. Hence, CLE requires the use of the intravenous contrast agent fluorescein (1.0–5.0 mL of 10%)., CLE was performed with either an endoscope-based confocal laser endomicroscopy (Pentax, Fort Wayne, NJ, USA; "eCLE") or a CLE probe (Cellvizio, Mauna Kea Technologies, Paris, France; "pCLE") that is negotiated *via* the accessory channel of regular endoscopes. However, eCLE is no longer available, even though the majority of confocal applications were studied using it (195). Studies suggest that CLE of intestinal inflammation in IBD can contribute to individualized therapy guidance and predict response and relapse (196). Furthermore, significant progress in molecular *in vivo* imaging may allow exploration of the pathophysiology of IBD and targeted therapies the therapy (197). In study by Li et al. a good correlation between CLE evaluation of crypt architecture and fluorescein leakage with histological findings in subjects with UC was observed. More than 50% of patients with mucosal healing detected during HD-WLE exhibited acute inflammation on histology, whereas no patients in remission confirmed by CLE demonstrated acute inflammation on histology (198). The same group evaluated whether CLE could be used to predict UC relapse in 43 patients with UC. The relapse rate among subjects with CLE-confirmed active disease was significantly higher compared to those with a non-active disease ($P < 0.001$) (199). CLE has also been studied to specifically determine gastrointestinal (GI) barrier function in patients with IBD (200). Physiologically, intestinal epithelial cells shed from the epithelial layer, whereas new cells migrate from the basal layers in crypts. This gap created by cell shedding can be visualized by CLE and serves as

a marker of increased permeability in IBD patients resulting in fluorescein leakage into the lumen (201). Kiesslich et al. observed a significant barrier dysfunction in 47 patients with UC and 11 patients with CD and showed a correlation between intestinal barrier dysfunction and increased risk of relapse (201). Similar results were obtained by Buda et al., who demonstrated that a composite score (Buda score) combining colonic fluorescein leakage with crypt diameter predicts disease flare within one year of follow-up (202).

Our own data suggest CLE-based real-time visualization of blood flow, vascular pattern, and mucosal changes allows an exact quantification of the level of inflammation in IBD. These criteria proved to be reliable to predict early therapy response in patients undergoing anti-integrin therapies (Vedolizumab, VDO) already after two weeks of treatment (203).

Imaging Cutaneous Inflammation by Optical Coherence Tomography (OCT)

Background

Optical biopsy is the concept to replace physical tissue sampling by optically investigating tissue *in vivo* to gain information on pathological changes. One promising approach is optical coherence tomography (OCT), which is a well-established imaging technique in ophthalmology (204). Analogous to ultrasound, OCT uses the reflection of light waves from different tissue interfaces. It measures the propagation time of light by interferometry instead of direct time-of-flight measurements and achieves a higher resolution than ultrasound. OCT is non-invasive, non-contact, fast, and needs no additional marker or contrast agents. Resolution is limited by the spectral band-width of the light source and NA of the imaging optics. Traditionally, most OCT systems provided a resolution of 5 micrometers or worse, which only resolves tissue layers and larger morphology but not cellular structures (205). Changes in these larger structures due to inflammatory processes can be visualized and quantified in cross-sectional or even volumetric images (206–208). Due to the use of interferometry in the imaging process, OCT also depicts very sensitively local motion. This enables a marker-free angiography which visualizes vessels down to the capillary level (209, 210).

Very high resolution OCT systems have been investigated in the past (211–215), but only recently their full potential has been demonstrated for cellular imaging (216). At a resolution better than 2 μm tissue structures on cellular and subcellular level become visible (216–218). Besides resolution, imaging contrast is also important. Contrary to fluorescence imaging, OCT lacks a cell-specific contrast. Neither are specific marker available. However, transferring the principle of OCT angiography to higher resolution and longer time scales, a cell and tissue specific contrast was introduced. It was first demonstrated with FF-OCT for en-face images (219, 220) and recently also using scanning OCT for cross-sectional imaging (221). The contrast is based on microscopy intra-cellular motion, which in general caused by structures below the imaging resolution, but is detected by the interferometric imaging process on which OCT is based. Combining microscopic resolution and dynamic motion contrast individual cells and connective tissue are visible with a fluorescent-like contrast (**Figure 7**). Since microscopic motion is the basis of contrast, general tissue motion destroys the contrast. Mechanical stabilization of the tissue is crucial and currently in-vivo imaging has yet been demonstrated. Analyzing cellular morphology and dynamic processes of immune cells may, in the future, enable a marker-free optical biopsy of inflammatory processes by OCT.

In ophthalmology, OCT has become standard for retinal diagnosis and is also clinically used for imaging the anterior segment of the eye. The unique properties of OCT which provides micrometer lateral and axial resolution despite the limited pupil size of the eye, make OCT the only imaging technique, which can visualize and quantify the layered structure of the retina. The retina offers unique optical access to neuronal tissue and the microcirculation and gives opportunity to diagnose and quantify systemic neuronal and vascular diseases. Diagnostic applications include inflammatory diseases like lupus, systemic sclerosis, Behçet disease, spondylitis, and familial Mediterranean fever (208). OCT and OCT angiography are also valuable tool in inflammation diagnosis of the anterior segment (222, 223). The clinical applications of OCT in areas other than ophthalmology are currently found in skin imaging, cardiovascular imaging, and gastroenterology, as only there clinically approved OCT devices are commercially available. Previously, most dermatology studies dealt with the visual presentation of tumor diseases and only few papers evaluated OCT's potential for inflammatory diseases (206, 224–226) In gastrointestinal diseases the first results from endoscopic optical biopsy were published 20 years ago (227). OCT could be used to identify transmural inflammation and morphological differentiation between UC and CD from patient ex vivo tissue samples (228) and *in vivo*, providing a valuable tool to distinguish CD from UC (229). This is especially relevant since biopsies are insufficient to assess for transmural inflammation. Although these data are encouraging, subsequent confirmation in larger, longitudinal follow-up trials is missing so far.

Definitely the potential of OCT is not yet exhausted in the field of inflammation. Especially, the significant increase in imaging speed and imaging resolution in last year gives new options for imaging inflammatory processes on a cellular level. A response to treatment could be detected early to enable individual adaptation of the treatment strategy through the accurate representation of the inflammatory processes. If molecular and cellular changes are detected at an early stage of disease progression or in the treatment of inflammatory diseases, in that case, it is possible to make and optimize individual treatment decisions. The following section will concentrate on dermatological applications of OCT, which is currently the only field in which commercial OCT devices with cellular resolution are available of clinical diagnosis (230).

Applications

The skin is the largest organ of the human body. In the clinical routine, the patient's skin is firstly examined with the naked eye. Conspicuous skin lesions can be further assessed with the help of dermoscopy, which allows the magnification of the skin surface and the superficial vessels. Skin alterations, including cellular and deep vascular changes, typically require tissue removal for histological

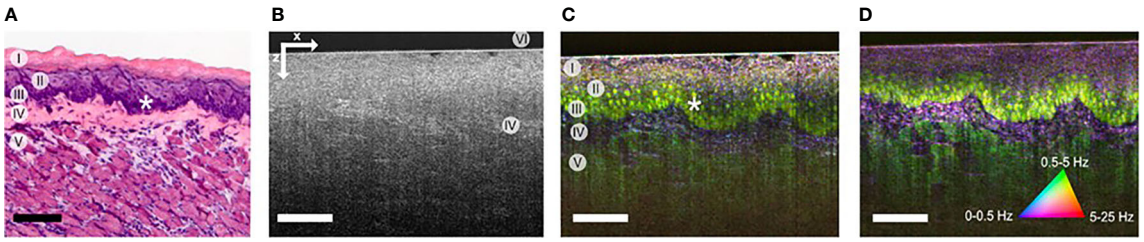

FIGURE 7 | **(A)** HE stained histology of the imaged sample at different location (I) cornified layer, (II) granular & spinous layers, (III) basal layers, (IV) lamina propria, (V) muscle, and (VI) glass plate. **(B)** OCT image of mouse tongue; lamin propira (IV) can be identified by brighter contrast. **(C)** Corresponding dynamic contrast mOCT image with a focus in basal layer (I-V) and even nuclei (*) are visible. **(D)** Dynamic contrast m OCT image with a focus in the lamina propria; the image size is 380x500 μm (zx); scale bar, 100 μm [from ref. (221)].

examination. Histology is the gold standard diagnostic method. However, invasiveness, expenditure of time, limitation to two-dimensional sectioning, and lack of monitoring dynamic changes make the histological examination evidently improvable. Hence, there is a demand for non-invasive methods that enable real-time, three-dimensional, and *in vivo* imaging of the skin.

OCT has the potential to combine fast bedside imaging with the opportunity to monitor therapeutic effects (224). There are reports on applications of OCT in dermatology mostly for skin tumors (231), but rarely for inflammatory skin diseases (232) and evaluation of treatment effects (233). Vascular alterations can be detected using OCT angiography, also known as dynamic OCT (234). Since 1997, reports related to OCT in dermatology have increased (235). This implicates the growing importance of OCT for clinical applications. Other well-established, *in vivo* imaging modalities that could be compared to OCT are ultrasound (236), confocal microscopy (237), multiphoton tomography (238), and magnetic resonance imaging (239). The highest comparability with regard to resolution and penetration depth is with OCT and high-frequency ultrasound. Both methods provide time- and cost-effectiveness. Also, OCT could be widely available in the future. The resolution typically decreases with higher penetration depth. Ultrasound imaging exhibits high tissue penetration visualizing fat and muscle, but the resolution is lower compared to OCT (236). Inflammation changes the tissue composition leading to higher water content and lower collagen content. Therefore, it has been shown for OCT that the signal penetration could even be increased due to lower scattering. In contrast, inflammatory processes and edema lead to a signal decrease for ultrasound (225).

Skin diseases that result in structural and vascular changes can be determined and quantified in OCT. For example, atopic dermatitis and plaque psoriasis are common inflammatory diseases that lead to a higher first intensity peak in the A-scan due to higher reflectivity (225). Further, the second intensity peak is correlated with alterations of the dermal-epidermal junction. The efficacy of potent biologic treatments could be assessed using OCT. Imaging parameters could be skin structure, epidermal thickness (**Figure 8**), entrance peak, dermal reflectivity, attenuation coefficient, plexus depth, vessel diameter, density, and tortuosity (**Figure 9**) (224, 240).

OCT can be applied for monitoring psoriasis treatment. In the near future, skin assessment with the use of OCT could become an inherent part of the clinical routine (233). Finally, the development of an OCT-based inflammation score system with regard to skin structure and perfusion could allow more tailored treatment opportunities. The goal of precision medicine is the gain of more predictive information from OCT data on early treatment response or treatment failure of current biologic treatments.

CONCLUSIONS

Imaging inflammation is a key component to understand and treat the various manifestations of the disease. Virtually all modern imaging technologies have identified inflammation as a goal and are developed methods to image it. The overview in this article shows that different modalities are needed to tackle different aspects of the disease. Impressive progress has been made and will continue, bringing precision medicine to life.

In tumor imaging and analytics, several new methods have been tested in clinics. These methods could be brought into use quite easily in many cases of inflammation disease as well. Like, cancer imaging techniques might solve problems that appear in inflammation imaging today. Overall the problem of optical imaging in medicine in humans is the shallow penetration. The solutions might include guiding the light deep into the body endoscopes or optical fibers and develop better imaging tracers. Also, photoacoustic might be a valuable method in future imaging. Optical 3D imaging offers the best platform for extending molecular imaging from cells to the tissue and organ level. OCT can be used to see micro-structures below the surface, scan larger areas, quantify inflammation by geometry measurements, and visualize blood flow. Thus OCT could be used to image the structural tissue changes in IBD. PET and SPECT, both being available for humans and rodents, can play a key role in translating the knowledge gained in preclinical research into the clinics. These two imaging techniques are being already regularly used in macrophage detection and, in the case of FDG-PET, also for energy consumption in inflammations; however, further opportunities are expected if more specific molecular targets with adequate radiolabeling are developed. All of the imaging modalities would benefit from new, better, and more specific tracers. New solutions in MRI would

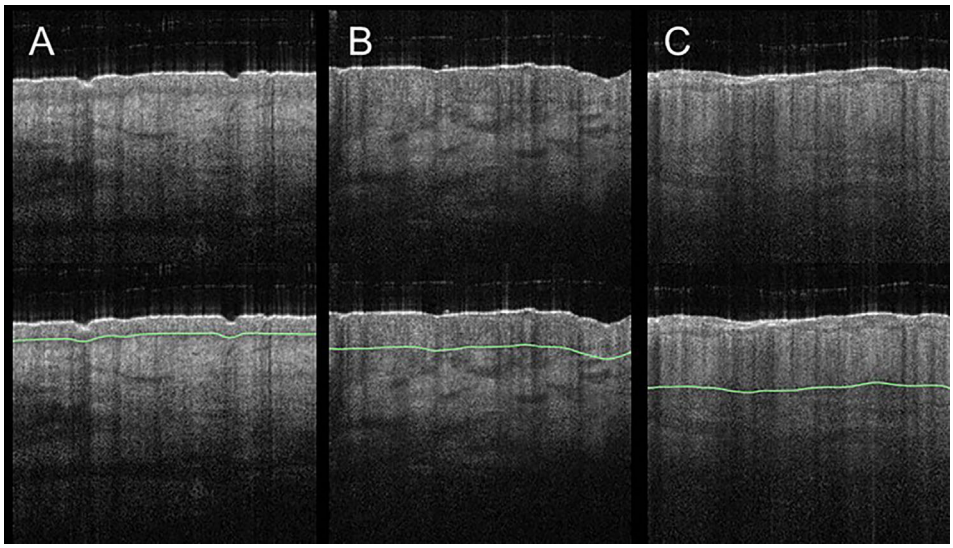

FIGURE 8 | Acute and chronic inflammatory skin diseases can lead to an increase of the epidermal layer. Compared to healthy skin **(A)**, involved skin in atopic dermatitis **(B)** and in plaque psoriasis **(C)** exhibit a thicker epidermal layer. Changes of epidermal thickness (green line) can be visualized in vertical B-scans and measured by OCT.

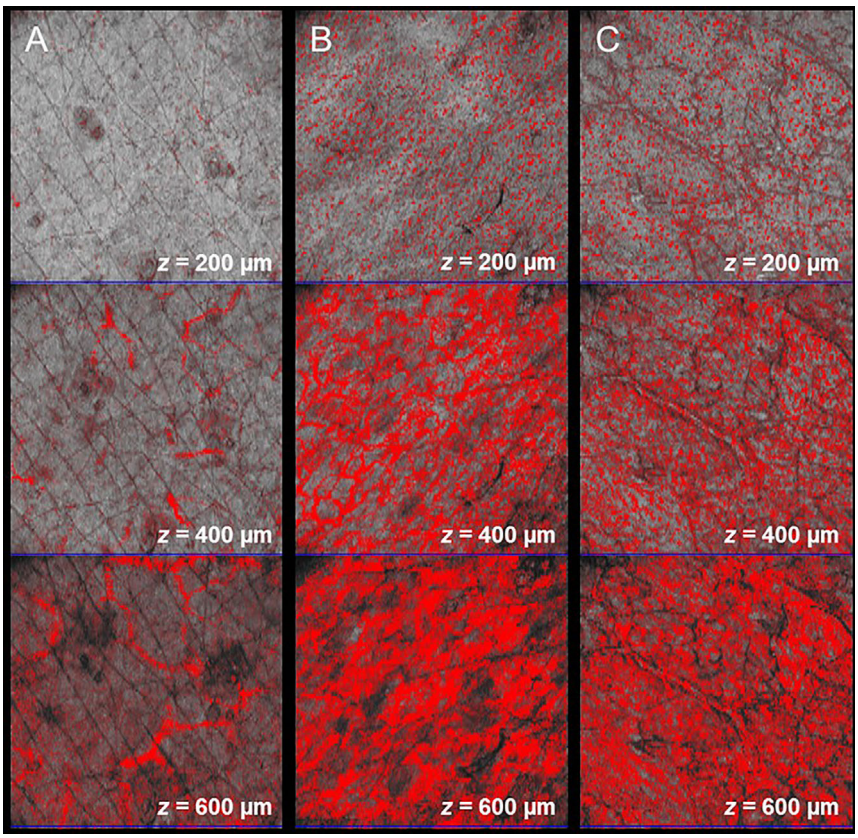

FIGURE 9 | Angiographic OCT allows the visualization of elongated capillary loops in the superficial papillary dermis and the underlying vessel plexus. In comparison to the healthy control **(A)**, changes of vascular pattern, vessel diameter, depth, and density can be observed in lesional skin in atopic dermatitis **(B)** and in plaque psoriasis **(C)**.

TABLE 1 | Current clinical and relevant assortment of potential preclinical tracers of different imaging modalities have been gathered to the table.

Technique	Disease/organ	Marker	Preclinical/Clinical	References number
ASL-MRI	stroke / brain	cerebral blood flow; arterial transit time	Clinical	(241–243)
	stroke / pediatric brain	cerebral blood flow	Clinical	(244–246)
	tumors / brain	cerebral blood flow	Clinical	(247, 248)
DCE-MRI	multiplesclerosis (MS)/Brain	blood-brain barrier permeability; volume transfer constant; extracellular space volume fraction	Clinical	(249)
	stroke / brain	blood-brain barrier permeability; contrast-agent leakage rate; volume transfer constant	Clinical	(250)
DCE-MRI	multiplesclerosis (MS)/Brain	BBB permeability	Clinical	(19)
	prostatic hyperplasia / prostate	perfusion fraction; extraction fraction; mean transit time; extravascular-extracellular volume	Clinical	(18)
DSC-MRI	tumors / brain	cerebral blood volume	clinical	(14, 15)
	stroke / brain	cerebral blood flow; cerebral blood volume; mean transit time	clinical	(243)
MRI	Vasculitis/central nervous system/ brain	arterial wall thickening, vessel wall enhancement on post-gadolinium black-blood MRI	Clinical	(33–37)
	Intracranial aneurysms/brain	aneurysm wall enhancement on post-gadolinium black-blood MRI	clinical	(42–46, 53–56)
	IBD / small bowel and colon	bowel wall thickening, restricted diffusion, edema, increased contrast media uptake, strictures, ulcerations, reduced motility, mesenterial reaction	clinical	(59–64, 66–69)
metabolic hyperpolarized MRI	arthritis	anaerobic glycolysis; lactate; lactate dehydrogenase	Preclinical/Clinical	(79)
	liver	anaerobic glycolysis; lactate; lactate dehydrogenase	preclinical	(80)
	myocardial infarction	anaerobic glycolysis; lactate; lactate dehydrogenase	Preclinical/Clinical	(81)
	muscullo skeletal	anaerobic glycolysis; lactate; lactate dehydrogenase	preclinical	(82)
	general inflammation	pH; bicarbonate/CO2, zymonic acid; pyruvate dehydrogenase	preclinical	(251)
gaseous hyperpolarized MRI	lung, brain	ventilation, dissolved-phase imaging; ^{129}Xe	clinical	(83)
	head, neck, lungs	FLASH) MRI; ^{3}He, proton (H_2O, CH_2-group)	pre-clinical/clinical	(84)
	COPD/lung, asthma, cystic fibrosis	ventilation, diffusion and dissoved-phase imaging; ^{129}Xe, ^{3}He; Oxygen concetration, lung capacity.	clinical	(85)
	lung, kidney, brain/ COPD	Ventilation, dissolved-phase imaging; ^{129}Xe, ^{3}He; barrier uptake, red blood cell transfer, ventilation defect percentage.	clinical	(86)
	lung/ emphysema	diffusion imaging; ^{3}He; apparent diffusion coefficient.	clinical	(87)
	lung/COPD, Idiopathic pulmonary fibrosis, left heart failure, pulmonary arterial hypertension	Ventilation and gas transfer maps ; ^{129}He; ventilation defects, Red blood cell- transfer.	clinical	(88)
	lung/ COPD	Transfer Contrast MRI; ^{129}Xe; apparent diffusion coefficient.	clinical	(89)
	lung/ COPD, asthma	3D dissolved-phase imaging; ^{129}Xe; red blood cell to tissue–plasma ratio.	clinical	(90)
	lung/ idiopathic pulmonary fibrosis	Spectroscopic imaging; ^{129}Xe; regional gas exchange.	clinical	(91)
	lung/ COPD, asthma	2D and 3D ventilation imaging, ^{129}Xe; dissolved-phase to gas-phase xenon ratio.	clinical	(92)
SPECT/PET				
	IBD /and many other inflammations	18F-FDG	clinical	(94, 102–117)
	IBD	Leukocytes99mTc-HMPAO-leukocytes	clinical	(252)
	IBD	CXCL899mTc-CXCL8	clinical	
	IBD	β764Cu-FIB504.64-Fab	Preclinical	
	IBD	α4β764Cu-DATK32	Preclinical	
	IBD	β764Cu-FIB504.64-Fab	Preclinical	
	IBD	β764Cu-FIB504.64-F(ab′)2 (fragments)	Preclinical	
	IBD	CD489Zr-GK1.5 cys-diabody	Preclinical	
	IBD	TNF-α99mTc-InfliximabRatsTNBS	Preclinical	
	IBD	IgG111In-IgGRabbitsTNBS	Preclinical	
	IBD	Leukocytes111In-WBC	Preclinical	

(Continued)

TABLE 1 | Continued

Technique	Disease/organ	Marker	Preclinical/Clinical	References number
	IBD	Liposomes111In-liposomes	Preclinical	
	IBD	IL-899mTc-HYNIC-IL-8RabbitsTNBS	Preclinical	
	IBD	Granulocytes99mTc-HMPAO-Granulocytes	Preclinical	
	IBD and many inflammations	imaging inflammatory cells	Preclinical	(253)
	IBD and many inflammations	11C-PK11195,	Preclinical	
	IBD and many inflammations	18F-FEDAA1106,	Preclinical	
	IBD and many inflammations	18F-FEMPA,	Preclinical	
	IBD and many inflammations	18F-GE-180,	Preclinical	
	IBD and many inflammations	68Ga-DOTATATE,	Preclinical	
	IBD and many inflammations	64Cu-DOTATATE,	Preclinical	
	IBD and many inflammations	68Ga-DOTANOC,	Preclinical	
	IBD and many inflammations	18F-FDR-NOC,	Preclinical	
	IBD and many inflammations	68Ga-DOTATOC,	Preclinical	
	IBD and many inflammations	64Cu-DOTA-DAPTA-comb nanoparticles, 64Cu-DOTA-ECL1i,	Preclinical	
	IBD and many inflammations	64Cu-DOTA-vMIP-II,	Preclinical	
	IBD and many inflammations	64Cu-vMIP-II-comb nanoparticles,	Preclinical	
	IBD and many inflammations	18F-FOL Folate receptor βMacrophages,	Preclinical	
	IBD and many inflammations	68Ga-NOTA-MSA,	Preclinical	
	IBD and many inflammations	18F-FDM,	Preclinical	
	IBD and many inflammations	64Cu-MMR and 68Ga-MMR nanobodies,	Preclinical	
	IBD and many inflammations	18F-fluorothymidine,	Preclinical	
	IBD and many inflammations	18F-fluoromethylcholine,	Preclinical	
	IBD and many inflammations	11C-choline,	Preclinical	
	IBD and many inflammations	68Ga-Fucoidan,	Preclinical	
	IBD and many inflammations	64Cu-DOTA-anti-P-selectin antibodies,	Preclinical	
	IBD and many inflammations	18F-4V,	Preclinical	
	IBD and many inflammations	64Cu-VCAM nanobody,	Preclinical	
	IBD and many inflammations	18F-HX4,	Preclinical	
	IBD and many inflammations	18F-FMISO,	Preclinical	
	IBD and many inflammations	62Cu-ATSM,	Preclinical	
	IBD and many inflammations	18F-fluciclatide,	Preclinical	
	IBD and many inflammations	18F-Galacto-RGD,	Preclinical	
	IBD and many inflammations	18F-Flotegatide,	Preclinical	
	IBD and many inflammations	64Cu-DOTA-C-ANF,	Preclinical	
	IBD and many inflammations	DOTA-CANF-comb nanoprobe,	Preclinical	
	IBD and many inflammations	18F-florbetaben,	Preclinical	
	IBD and many inflammations	18F-flutemetamol,	Preclinical	
	IBD and many inflammations	68Ga-DOTATATE,	Preclinical	
	IBD and many inflammations	18F-FET-βAG-TOCA	Preclinical	
Optical/PAI	rheumatoid arthritis	ICG blood flow indicator	Clinical	(131)
		Many tracers examples aMSH, MMP binding tracer, RGD, VEGF targeting nanoparticles, ASMase targeting liposome, Alendronate targeted nanoparticles	Preclinical	(133–143)
	IBD	Hemoglobin	preclinical	(168)
	CD	Hemoglobin and fibrosis	preclinical	(169–171)
	Arthritis	L-selectin/P-selectin-targeting contrast agent	preclinical	(172)
	Arthritis	Hemoglobin	preclinical	(172–175)
	Atherosclerosis	gold nanorods conjugated with MMP_2 antibody	preclinical	(140)
	Wound	Endogeneous bacterial fluoresence	Preclinical	reviewed in (158)
	Invasive- and biomaterial-associated bacterial infections	conjugated vancomycin to IRDye	Early clinical trial	(161, 167)
	Tuberculosis	fluorogenic substrates for beta-lactamase	preclinical	(164)
	Wound infection	Lectin base fluorescent nanoparticle	preclinical	(165)
	Bacterial infections (implants)	Antimicrobial peptide conjugated to a radioisotope and a fluorescent dye	preclinical	(166)
	Not limited to certain organs or diseases. Can be used to measure morphological changes of tissue.	Fluorecenct stains and/or autofluorecence	Certified medical devices for in vivo skin measurements avaible.	(184)

(Continued)

TABLE 1 | Continued

Technique	Disease/organ	Marker	Preclinical/Clinical	References number
			Other applications are preclinical.	
Digital chromoendoscopy	IBD/colon	Mucosal surface patterns	Clinical	(191, 192)
Endoscopic ultrasound	IBD/colon	Total wall thickness, mucosal vascularity	Clinical	(193, 194)
Confocal laser endomicroscopy	IBD/colon	Crypt diameter, fluorescein leak, mucosal vascularity	Clnical	(195–203)
TPM	Not limited to certain organs or diseases. Can be used to measure morphological changes of tissue.	Fluorecenct stains and/or autofluorecence	Certified medical devices for in vivo skin measurements avaible. Other applications are preclinical.	(184, 236, 238)
OCT	Human eye		Clinical	(204, 205, 208, 210, 222, 223),
	Human coronary artery		Clinical	(205, 216)
	Human oesophagus		Clinical	(205, 207)
	Human small intestine		Clinical	(205, 207)
	Human colon		Preclinical	(205, 207, 214, 228, 229)
	Human biliary and pancreatic ducts		Clinical	(205, 207)
	Human lung		Preclinical	(205, 217, 218)
	Skin		Clinical	(205, 212, 224–226, 230–235, 239, 240)
confocal microscopy	skin		Clinical	(233, 237)

serve the possibility to track inflammation based on metabolic activity differences between normal and inflamed tissues.

A combination of different imaging modalities would simultaneously offer information about the structure, success of targeting, or metabolic activity from the tissue of interest. In fact, multimodality has already reached the clinical and preclinical environments, e.g. through PET/CT and PET/MRI. Further benefits could be achieved by developing bi- or even multimodal imaging agents. Although bimodal probes have been proposed for PET/MR as well as for PET and optical imaging, they still remain less specific than the probes designed for each single modality. In addition to diagnostic purposes, imaging could be an extra tool in medical operations like in surgery. There are indeed several concepts for intraoperative optical imaging like optical fluorescent operational microscopes, fluorescent cameras for robotic surgery, OCT integrated surgical microscopes, as well as methods based on radioactive signal detection. Among the latter, gamma, beta minus, and beta plus probes, mini gamma cameras, intraoperative PET detectors and freehand SPECT have proposed. We have gathered the current clinical and relevant assortment of potential preclinical tracers of different imaging modalities as a table (**Table 1**). Some of these tracers, especially nano-probes, could be easily converted to multimodal tracers. While some of these methods have already reached the clinical arena, some others are still under investigation. Here additional information collected by different imaging modalities would help to solve multiple current unmet needs in inflammation like in robotic real-time surgery operations.

The newly-developed research techniques are tested in clinical and preclinical studies to improve diagnosis and identify the individual patient response to treatment at an early stage in precision medicine. The close cooperation of engineering expertise with clinical applications leads to further developing state-of-the-art imaging methods in inflammation medicine. Modern optical microscopy already enables microscopically small cell changes to be identified and assessed in real-time. The existing experimental methods can only be transferred into a clinical application with direct benefits for the patient by close dialogue between the different scientific disciplines.

AUTHOR CONTRIBUTIONS

RH, GH, OP, TP, and JH wrote the optical imaging part. RH, GH, J-BH, MR, OP, and TP wrote abstract, introduction, and conclusions and did proofreading for the review. TP put the text together and was a contact person for the writers. JEH, LH, RH, GH, and ME made the OCT part. JK, GH, and RH provided the TP part. MR and OP wrote PET/SPECT part. J-BH, MB, NL, PU, AF, MSP, and MA provided the MRI part. ME contributed writing the endoscopic approaches in inflammatory bowel

disease. All authors contributed to the article and approved the submitted version.

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