PRECISION MEDICINE AND IMMUNO-MEDIATED INFLAMMATORY DISEASES: LATEST PROGRESS AND NEXT CHALLENGES

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Personalized Medicine of Monoclonal Antibodies in Inflammatory Bowel Disease: Pharmacogenetics, Therapeutic Drug Monitoring, and Beyond

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The pharmacotherapy of inflammatory bowel diseases (Crohn's disease and ulcerative colitis) has experienced significant progress with the advent of monoclonal antibodies (mABs). As therapeutic proteins, mABs display peculiar pharmacokinetic characteristics that differentiate them from chemical drugs, such as aminosalicylates, antimetabolites (i.e., azathioprine, 6-mercaptopurine, and methotrexate), and immunosuppressants (corticosteroids and cyclosporine). However, clinical trials have demonstrated that biologic agents may suffer from a pharmacokinetic variability that could influence the desired clinical outcome, beyond primary resistance phenomena. Therefore, therapeutic drug monitoring (TDM) protocols have been elaborated and applied to adaptation drug doses according to the desired plasma concentrations of mABs. This activity is aimed at maximizing the beneficial effects of mABs while sparing patients from toxicities. However, some aspects of TDM are still under discussion, including time-changing therapeutic ranges, proactive and reactive approaches, the performance and availability of instrumental platforms, the widely varying individual characteristics of patients, the severity of the disease, and the coadministration of immunomodulatory drugs. Facing these issues, personalized medicine in IBD may benefit from a combined approach, made by TDM protocols and pharmacogenetic analyses in a timeline that necessarily considers the frailty of patients, the chronic administration of drugs, and the possible worsening of the disease. Therefore, the present review presents and discusses the activities of TDM protocols using mABs in light of the most recent results, with special attention on the integration of other actions aimed at exploiting the most effective and safe therapeutic effects of drugs prescribed in IBD patients.

Keywords: inflammatory bowel disease, monoclonal antibodies, pharmacokinetics, interindividual variability in drug response, therapeutic drug monitoring, pharmacogenetics

INTRODUCTION

The therapy of inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), has been based on aminosalicylates, antimetabolites (i.e., azathioprine, 6-mercaptopurine, and methotrexate), and immunosuppressants (corticosteroids and cyclosporine). These drugs may control symptoms and signs of IBD at the cost of both systemic toxicities and treatment failures observed in a variable percentage of patients (Saibeni et al., 2008; Wahed et al., 2009; Jeong et al., 2019). These issues motivated the scientific community to search for newer pharmacological entities, including monoclonal antibodies (mABs). Thanks to their specific activity against inflammatory processes and their tolerability, mABs represent an area of intense research (Dulai and Sandborn, 2016; Yamamoto-Furusho, 2018; Katsanos et al., 2019).

The clinical use of mABs has shed light on their pharmacokinetic characteristics; a relatively small volume of distribution (approximately equal to plasma and interstitium), a clearance depending on several processes, a negligible renal excretion, and the presence of antidrug antibodies (ADAs) make the pharmacokinetics of mABs of particular interest for interindividual variability, which in turn may depend on genetic polymorphisms. Therefore, the present review will discuss the factors that can affect drug pharmacokinetics, the application of therapeutic drug monitoring (TDM), the role of pharmacogenetic analyses, and their possible integration in the context of personalized medicine for IBD.

MONOCLONAL ANTIBODIES USED IN INFLAMMATORY BOWEL DISEASES

The first mABs used in IBD were designed to target the pathway of tumor necrosis factor α (TNFα), which controls cell proliferation and differentiation and promotes a proinflammatory response. Infliximab, adalimumab, golimumab, and certolizumab pegol are prescribed in moderate to severe forms of IBD that respond poorly to other therapies in both induction and maintenance. Indeed, they may ameliorate disease control, reduce hospitalizations and surgery, and finally improve quality of life. Although these are beneficial therapeutic effects, patients may experience a relapse of the disease (Casanova et al., 2017; Bots et al., 2019). The causes behind the failure are not well understood, but individual changes in drug pharmacokinetics and pharmacodynamics or immunogenicity represent possible risk factors. For these reasons, TDM protocols guide dose optimization for every patient on an individual basis.

More recently, mABs can also target extracellular proteins involved in the onset and maintenance of bowel inflammation so it is understood that the number of drugs for the treatment of IBD will increase over the next few years (Hindryckx et al., 2018). In particular, vedolizumab impedes the binding of α4β7-integrin expressed on memory T cells to the mucosal addressin cell adhesion molecule-1 (Mad-CAM-1). The drug is an appropriate therapeutic alternative in IBD patients who developed systemic infections after immunosuppressant regimens or in older patients due to its specific tissue targeting capability for inflammatory processes within gut mucosa (Colombel et al., 2017). Ustekinumab binds the p40 subunit of IL12 and IL23 and blocks the activation of $CD4^+$ lymphocytes by activating APCs and their subsequent differentiation into Th1 and Th17 cells (Lamb and Duggan, 2017). As a consequence, the inflammatory cascade through the synthesis and release of several distinct cytokines (i.e., IFNγ, IL2, IL10, IL22, TNFα, and TNFβ) is reduced.

Therapeutic Monitoring of Monoclonal Antibodies in Inflammatory Bowel Diseases

As presented and discussed in the next sections, many factors may significantly influence and alter the pharmacokinetics of mABs in IBD patients, including possible causes of suboptimal efficacy to treatment or a frank loss of response. TDM protocols may overcome these issues by measuring minimum plasma concentrations (C_{min}) and subsequently comparing the values with therapeutic ranges associated with the clinical efficacy of the mABs as defined in clinical trials. In other cases, the therapeutic window of plasma concentrations reflects the improvement in endoscopic endpoints (i.e., mucosal healing) or biomarkers of inflammation, as well as C-reactive protein (CRP) and fecal calprotectin (FCP). In general, the control of the disease in the early phase of therapies (the induction phase) requires higher trough plasma concentrations than in the following postinduction and maintenance phases.

Among the mABs used in IBD, infliximab displays the most extensive collection of results. The suggested lower limit of the therapeutic range of C_{min} values is ≥ 20 mg/L in the induction phase at week 2 (Papamichael et al., 2019a), even if the achievement of mucosal healing in the first weeks of treatment depends on higher plasma concentrations (≥25 mg/L) in both UC and CD patients. That threshold progressively diminishes at week six of therapy (≥10 mg/L, postinduction phase) and, finally it is ≥ 3 mg/L in the postinduction (week 14) and maintenance phases (Vande Casteele et al., 2015), as obtained in CD and UC patients. These trough levels are associated with endoscopic and clinical remission, as well as CRP normalization (≤5 mg/L). However, higher threshold values (≥7 mg/L) at week 14 were associated with clinical remission at weeks 14 and 54 (Kennedy et al., 2019) and mucosal healing (Ungar et al., 2016; Yarur et al., 2017; Papamichael and Cheifetz, 2019). In children, the lower bound of the therapeutic range for trough values of infliximab is 29, 18, and 5.4 mg/L at two, six and ≥14 weeks of treatment, respectively (van Hoeve et al., 2018a, 2018b; Clarkston et al., 2019).

In agreement with these findings, C_{min} values of adalimumab should be higher than 5 mg/L as suggested by several studies (Mazor et al., 2014; Bodini et al., 2016; Nakase et al., 2017), while for mucosal healing and histologic remission, a target range of 8–12 mg/L is recommended (Ungar et al., 2016). Again, C_{min} values of ≥12 mg/L at week 14 predicted clinical remission at both weeks 14 and 54 (Kennedy et al., 2019).

For certolizumab pegol, a former study found C_{min} values > 7.6 mg/L in patients who achieved clinical remission (Colombel et al., 2014), but mucosal healing required higher concentrations

(i.e., >19.2 mg/L). More recently, studies identified higher threshold values in the postinduction (>23–36 mg/L) and maintenance phases (>14 mg/L) that were associated with clinical and endoscopic remission and CRP and FCP normalization in CD patients (Vande Casteele et al., 2018; Papamichael and Cheifetz, 2019).

Golimumab needs trough levels >2.5 mg/L at week 6, while a value of ≥1 mg/L is appropriate during maintenance in UC patients (Adedokun et al., 2017). Moreover, trough levels >8.9 mg/L at week 2 (induction phase) predict clinical response (at week 6). In CD patients pretreated with anti-TNFα drugs enrolled in a recent trial, subjects with mucosal healing had golimumab trough levels significantly higher than those who failed to respond (8.9 mg/L vs. 5.08 mg/L, respectively) (Boland et al., 2020); hence, the threshold of golimumab trough concentration was set at 8 mg/L in maintenance therapy.

In the case of vedolizumab, trough values > 28 mg/L at week 2 (and >24 mg/L at week 6) of treatment resulted in clinical response and mucosal healing in the induction phase of UC and CD patients (Williet et al., 2017; Dreesen et al., 2018; Papamichael and Cheifetz, 2019), a threshold value that becomes >20 mg/L in the maintenance phase (week 22) (Dreesen et al., 2018). The probability of clinical remission in both CD and UC patients increased to higher C_{min} values (95%CI, 35–84 mg/L) achieved at week 6 (Rosario et al., 2017). A more recent meta-analysis found a significant association between vedolizumab trough concentrations (>20 and >12 mg/L at week six and at maintenance, respectively) and therapeutic outcomes in UC patients but not in CD individuals (Singh et al., 2019). Of note, some studies questioned the usefulness of vedolizumab TDM (Pouillon et al., 2019) because data are heterogeneous and impede the definition of a clear therapeutic range, while the drug has a low potential for immunogenicity.

Finally, target C_{min} values of ustekinumab decrease from >4 mg/L in the postinduction phase to >0.8 or >1.4 mg/L (depending on the schedule of drug administration) in the maintenance phase (24 and 40 weeks of treatment) (Adedokun et al., 2018). Furthermore, trough concentrations ≥4.5 mg/L were associated with mucosal healing during maintenance (Papamichael and Cheifetz, 2019).

Overall, the concomitant presence of (persistent and rather transient) ADAs can explain the unexpected lower C_{min} values of mABs, as a result of an accelerated clearance. According to proposed algorithms, in the presence of a low ADA titer, patients may continue with the treatment while tailoring the dose to achieve plasma concentrations in the therapeutic range (Derijks et al., 2018). On the contrary, a high ADA titer recommends the prescription of immunomodulatory drugs, the substitution of the current mAB with another, or the switch toward a different drug class (Strik et al., 2017).

It is worth noting that two independent population pharmacokinetic studies found that the titer of ADAs correlated with the estimated clearance values of infliximab (Brandse et al., 2017) and vedolizumab (Okamoto et al., 2020) better than a simple dichotomic presence/absence result. These findings are likely suggesting that a more accurate segmentation of patients according to the ADA titer could further improve the individualization of therapeutic regimens.

Pharmacokinetics

Table 1 reports the pharmacokinetic characteristics of mABs currently used in the treatment of UC and CD patients (Klotz et al., 2007; Ordás et al., 2012). The molecular weight and structure of mABs influence the passage of drugs across cell membranes during absorption, tissue diffusion, and excretion. The convective transport through a hydrostatic/oncotic gradient between compartments and a sieving effect, which is determined by both the endothelial permeability and the size of mABs, is responsible for transmembrane transport (Ryman and Meibohm, 2017). For example, the diffusion of mABs is higher in the liver and bone marrow (which have sinusoids and fenestrated capillaries, respectively) than that in muscle and skin (characterized by low-permeable capillaries) (Cao et al., 2013). Moreover, in some tissues, reduced convective transport may decrease mAB diffusion (Cooper et al., 2013) so that transcytosis may ensure drug passage through cell membranes and barriers. Transcytosis depends on the binding of mABs to the neonatal Fc receptor (FcRn) expressed on endothelial cell surfaces, and it plays a (minimal) role even in the absorption of mABs after subcutaneous injection (Zhao et al., 2013) thanks to lymphatic vessels. Of note, the presence of an extracellular matrix does delay both the absorption and diffusion of mABs. However, the uptake of drugs by lymphatic vessels depends on lymph flow rate, gradients, and sieving coefficients, resulting in time to peak values between 2 and 8 days for adalimumab, golimumab, and certolizumab pegol, while bioavailability falls in the range 53–80% (Baumann, 2006; Deepak and Loftus, 2016). The loss of mABs during absorption may depend on several factors, as well as degradation within lymph nodes, and cellular uptake mediated by immunoglobulin receptors or due to Fab binding to its target antigen. The latter two mechanisms also account for mAbs excretion together with pinocytosis, as discussed below.

The mABs bind cell surface receptors or FcγR expressed by many immune cells as well as macrophages, monocytes, NK cells, and neutrophils (Gessner et al., 1998; Hayes et al., 2016). Then, endocytotic internalization brings the drugs within the cytoplasmic endosomes that fuse with lysosomes and mABs are inactivated. The same occurs when the Fab domain binds its target, the so-called target-mediated drug disposition (or TMDD) (Liu, 2018). The TMDD process is saturable at the therapeutic doses of mABs; hence, it does not play a pivotal role in the clearance of these drugs even if it is responsible for a portion of the variable pharmacokinetic profile of biologics. On the contrary, the uptake of mABs by endothelium (the pinocytosis process) seems to have a significant influence on the systemic clearance of mABs because of the larger endothelial surface area in the body, especially in gut, muscle, and skin (Wright et al., 2000).

It is worth noting that the neonatal FcR (FcRn), also known as Brambell receptor, is a salvage pathway for mABs. Indeed, after internalization, FcRn binds IgG and mABs at the acidic pH of the lysosome, so the complex is excluded from proteolysis and directed back to the cell membrane, where the

TABLE 1 | Main pharmacokinetic characteristics of mABs used in UC and CD patients.

	Infliximab	Adalimumab	Golimumab	Certolizumab pegol	Vedolizumab	Ustekinumab
Bioavailability	–	64%[a]	51%[a,H]	80%[a]	–	57%[a,CD]
T_{max} (d)	<1 h	5.46 ± 2.3[b], 8[c,H]	2–6[d], 1–7[H]	2.3–7.1	<1 h	7–8.5 [CD]
Vd (L)	4.5–6.0	4.7–6.0, 7.87[c,H]	4.1–8.8	7.6	2.73–3.28[e,H], 4.84	4.62 [CD]
$T_{1/2}$ (d)	7.8–13.7[c]	10–20, 16.8[c,H]	10.9[H]	14	15.1–22[e], 25.5	19–21 [CD]
Clearance (ml/h), [L/d]	15.3–18.4, (0.4–10.6)[c]	11–15	20.1 ± 5.8	14.3–19.5	6.5–6.6, (0.136–0.164[e,H]), (0.159[UC], 0.155[CD])	4.6 [CD], (0.19) [CD]
References	Derijks et al. (2018); Hemperly and Vande Casteele (2018); Berends et al. (2019)	Baumann (2006); Sánchez-Hernández et al. (2020), Berends et al. (2019)	Xu et al. (2010); Derijks et al. (2018)	Quetglas et al. (2015); Derijks et al. (2018)	Battat et al., 2019; Berends et al. (2019); EMA (2018)	Lamb and Duggan, 2017

Parameter values are expressed as:
[a]*subcutaneous injection;*
[b]*mean ± standard deviation;*
[c]*median;*
[d]*range;*
[e]*mean.*
CD, Chron's disease; UC, ulcerative colitis; H, healthy volunteers.

immunoglobulin is newly released in the extracellular space (Israel et al., 1996; Junghans and Anderson, 1996; Liu, 2018). In this manner, the FcRn counteracts the lysosomal degradation of approximately two-thirds of the IgG and mAB (Kim et al., 2007), and the terminal half-lives of therapeutic mABs (10–25 days) are similar to those of endogenous IgG (21 days) in the absence of further confounding factors (Rosario et al., 2015). The only partial exception to this mechanism pertains to certolizumab pegol because it lacks two domains of the constant region (C_{H2} and C_{H3}) that bind the receptor. However, the pegylation of certolizumab blocks the glomerular filtration and shields the mAB fragment from the uptake by the reticuloendothelial system (RES). Thanks to these characteristics, certolizumab pegol has a terminal half-life that is comparable with that of other mABs.

The glycosylation pattern of the carbohydrate chains at the Asn297 amino acid within the C_{H2} domain may influence both the pharmacokinetics and pharmacodynamics of mABs (Boune et al., 2020). In particular, mABs characterized by a low content or absence of fucose or sialic acid have shorter terminal half-lives, as well as those harboring a high mannose glycan content. These characteristics partly reflect the effect of glycosylation pattern on FcyR receptor binding and, as a consequence, on drug pharmacodynamics through the enhancement of ADCC and CDC. However, a study achieved different results by using two engineered yeast systems that produced an afucosylated mAB and its variant lacking glycosylation (Liu et al., 2011). Indeed, the *in vitro* binding affinity for FcRn was similar for both mABs and did not differ when compared with that of the same mAB synthesized in CHO cells. Interestingly, when injected in transgenic mice harboring the human FcRn, mABs produced in yeast systems shared the same pharmacokinetic characteristics. Overall, those findings suggested that glycosylation seemed incapable of influencing mAB-FcRn binding at least in those models.

The immunogenicity of the humanized or human IgG1-like mABs used for IBD is still present, and the production of ADAs is another cause of accelerated clearance. For this reason, TDM protocols consider both the drugs and the corresponding ADAs (see below).

Finally, the high molecular weight excludes mABs from filtration, but renal glomeruli can filter minor fragments, which are reabsorbed and metabolized in the extracellular space surrounding the proximal tubule (Waldmann et al., 1972).

The last note regards the distribution of mABs. As mentioned above, the physicochemical characteristics of the drugs, the endothelium permeability, and the presence of extracellular matrix proteins may affect the tissue diffusion of mABs. Overall, the volume of distribution (Vd) is in the range 8–20 L, thus approaching the extracellular body water (Dirks and Meibohm, 2010). Of note, Vd of vedolizumab and ustekinumab ranges between 4 and 5 L (Rosario et al., 2015; Deepak and Loftus, 2016).

Pharmacokinetic Variability

Several studies and population pharmacokinetic models evaluated the causes of variability between and within IBD patients to identify subgroups at higher risk of treatment failure and consequently candidates for treatment optimization (**Table 2**). Of note, some of those factors are changing over time (due to progressively better control of the inflammatory process), and modeling is carefully considering this characteristic (Vande Casteele et al., 2017). However, further factors may explain part of that pharmacokinetic variability as in the case of genetic variants of FcRn or the genetic background of patients. Therefore, a mixed pharmacokinetic and pharmacogenetic approach would be more attractive.

Absorption and Distribution

The absorption through the subcutaneous route may be influenced by changes in subcutaneous composition, blood perfusion, and lymph flow rate (Gibney et al., 2010). For

TABLE 2 | Factors significantly associated with changes in mABs clearance, increase ([+]) or decrease ([–]), according to findings from population pharmacokinetic studies.

Covariates	Infliximab	Adalimumab	Golimumab	Certolizumab pegol	Vedolizumab	Ustekinumab
Serum albumin	(–)[a]		(–)[a]	(–)	(–)	(–)
ADA	(+)[b]	(+)	(+)[a]	(+)	(+)[b]	(+)
Immunomodulators	(–)		(–)MTX			
Body weight	(–)[a]/(+)WT	(+) (WT or BMI or LBW)	(+)	(+) (BSA)	(+)	(+)
Inflammation markers		FCP(+)	CRP [+]	CRP (+)	CRP (+), FCP(+)	
Other	Sex (–)[F], ESR (+)[c]	UDASC, PEN	ALK (+)		Age (+), previous anti-TNFα (+)	Sex (+)[M]
References	Brandse et al. (2017); Hemperly and Vande Casteele (2018); Santacana et al. (2020); Bauman et al. (2020)	Sharma et al. (2015); Vande Casteele et al. (2019); Sánchez-Hernández et al. (2020)	Xu et al. (2018); Berends et al. (2019); Dreesen et al. (2020); Adedokun et al. (2020)	Wade et al. (2015)	Rosario et al. (2015); Osterman et al. (2019); Okamoto et al. (2020)	Xu et al. (2020)

Abbreviations: ADA, antidrug antibodies; ALK, alkaline phosphatase; BMI, body mass index; BSA; body surface area; CRP, C-reactive protein; ESR; erythrocyte sedimentation rate; FCP, fecal calprotectin; LBW, lean body weight; MTX, methotrexate; PEN, pen device (40 or 80 mg); UDASC, unexplained decline in adalimumab serum concentrations.
Female ([F]) or male ([M]) patients;
[a]also in pediatric patients;
[b]plasma ADA titration;
[c]only in pediatric patients.

example, UC patients weighing >100 kg or ≤100 kg had the same exposure after administration of golimumab at doses of 90 mg or 45 mg, respectively (Sandborn et al., 2008, Sandborn et al., 2012). Moreover, multivariate analysis identified BMI as a factor influencing adalimumab PK in a dose-escalation study (Bultman et al., 2012). The difference in doses did not correspond to a similar well-defined change in body weight, meaning that a single factor may only explain part of the interindividual variability. Indeed, as anticipated above, the administration of mAB at therapeutic doses may saturate the FcRn-dependent passage of the mAB from the injection site into the lymphatic vessels (McDonald et al., 2010) and the subsequent diffusion from blood to tissues.

From a purely pharmacokinetic point of view, body weight remains the most important factor associated with the variability in Vd (Rosario et al., 2015). Indeed, population pharmacokinetic studies identified body weight as the covariate exerting a significant effect on distribution of mABs used in IBD in both adult patients (Hemperly and Vande Casteele, 2018; Adedokun et al., 2020; Xu et al., 2020) and children (Sharma et al., 2015; Xu et al., 2018; Bauman et al., 2020). Sex may be an additional significant factor for Vd (Ternant et al., 2008; Fasanmade et al., 2009) as well as previous treatments with anti-TNFα mABs (Dreesen et al., 2020) and race and body surface area (which recalls patient's body weight) (Wade et al., 2015).

Elimination

As anticipated above, the elimination of mABs depends on the interplay of different mechanisms, and many of them are related to the severity of inflammatory status.

The increased concentration of endogenous IgG in severe, active disease may have opposite effects on mABs pharmacokinetics. Indeed, high concentrations of endogenous IgG may compete with mABs to bind with both FcRn (Morell et al., 1970) and FcyR, resulting in a shortened half-life of mABs or a rise in their plasma concentrations. Moreover, the binding of mABs to FcRn is species-specific so that further variability is expected when comparing different mABs.

The FcRn gene harbors a polymorphism consisting of variable number tandem repeats (VNTRs), with a decreased receptor expression associated with the VTNR2 allele at the cellular level. In turn, this leads to a diminished systemic exposure (as the area under the curve) of both infliximab (−14%) and adalimumab (−24%) in heterozygous VNTR3/2 IBD patients in comparison to VNTR3/3 homozygotes during induction (Billiet et al., 2016). These results suggest that FcRn has an effect on mAB absorption from subcutis.

Interestingly, higher serum concentrations of albumin correlated with reduced infliximab clearance (Fasanmade et al., 2009) and with increased systemic exposure to the drug (Fasanmade et al., 2010) (**Table 2**). Moreover, increased clearance of vedolizumab and ustekinumab corresponded with reduced values of serum albumin (Rosario et al., 2015; Lamb and Duggan, 2017). The reason of these relationships could be the competition for FcRn binding during the elimination process or the higher loss of proteins (including mABs) in the presence of the most severe inflammatory status.

Other markers of disease severity and inflammation may predict changes in mAB pharmacokinetics. Elevated serum concentrations of CRP were associated with increased clearance of both certolizumab pegol (Wade et al., 2015) and golimumab (Adedokun et al., 2020) and with lower plasma concentrations of ustekinumab (Lamb and Duggan, 2017). FCP correlated with the increased clearance of adalimumab (Sánchez-Hernández et al., 2020) and vedolizumab (Osterman et al., 2019), while erythrocyte sedimentation rate significantly influenced the clearance of infliximab in children (Bauman et al., 2020) (**Table 2**). Finally, a study reported the passage of infliximab into the gut lumen through the inflamed mucosa by

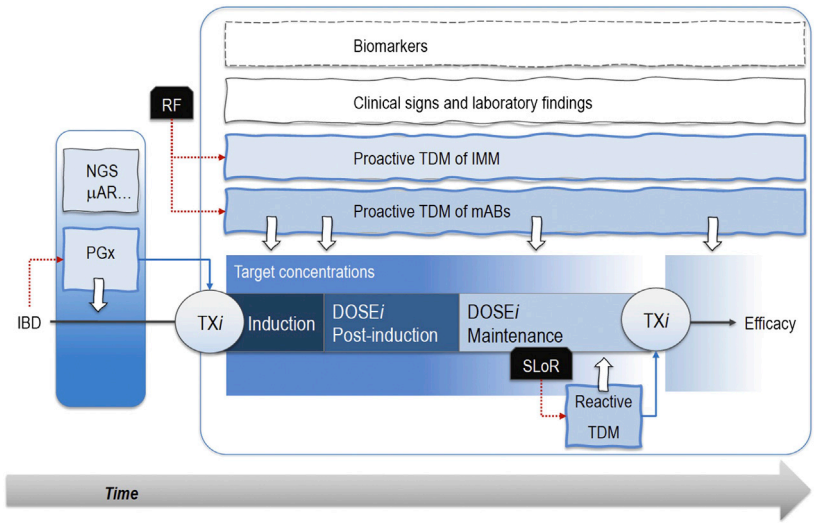

FIGURE 1 | Pharmacogenetic analyses (PGx) through next-generation sequencing (NGS) or microarrays (µAR) allow patients' segmentation and personalized pharmacological therapies (TXi) early in the induction phase. Notably, known risk factors (RF) associated with treatment failure sustain proactive therapeutic drug monitoring (TDM) protocols based on the measurement of drug plasma levels. Therefore, patients receive optimized drug doses (DOSE*i*) in the post-induction and maintenance phases when proactive TDM still mantains its role. The occurrence of a secondary loss of response (SLoR) triggers a reactive TDM that may support the decision about a possible change in drug regimen. These approaches take advantage of TDM of immunomodulatory drugs (IMM), clinical signs, laboratory findings, and biomarkers (i.e., C-reactive protein, fecal calprotectin, TNFα serum concentrations). Dotted red lines are the actions emerged from a critical event (i.e., the IBD diagnosis or SLoR), while the dotted blue lines represent the following therapeutic choices. White arrows are the analyses for PGX or TDM protocols.

an unknown mechanism (Brandse et al., 2015), as demonstrated in patients affected by the most severe UC (Kapel et al., 1992).

ADA in patients' plasma may be associated with poor treatment efficacy, while their production may be variable among the anti-TNFα agents, ranging from ≤2.3% for ustekinumab (Deepak and Loftus, 2016; Feagan et al., 2016), up to 25.3% for infliximab (Thomas et al., 2015), with intermediate percentage values for adalimumab (14.1%), certolizumab (6.9%), and golimumab (3.8%). Furthermore, the concomitant administration of other drugs, such as azathioprine and methotrexate, may modulate the production of ADA. Indeed, the infliximab-AZA combination was associated with a reduced incidence of ADAs (0.9% *vs.* 14.6%), increased C_{min} values of mAB, and a higher rate of corticosteroid-free remission rate than the sole infliximab (Seow et al., 2010). The combinations infliximab-thiopurines and adalimumab-methotrexate brought ADAs to undetectable levels in 77% of patients who previously experienced a loss of response due to immunogenicity (Strik et al., 2017). Even in children, methotrexate significantly reduced the clearance of infliximab (Xu et al., 2018) (**Table 2**), likely reducing the ADA production.

Of note, the formation of ADAs may depend on the schedule of the mAB regimen. Indeed, the occurrence rate of ADAs is higher after an occasional administration of mABs rather than a regular regimen (Colombel et al., 2010; Khanna et al., 2013). Moreover, the genotype at the HLA-DQA1*05 locus predicts the magnitude of the immune response against infliximab or adalimumab (Sazonovs et al., 2020). This finding may help in defining a combined signature to screen IBD patients who are candidates to receive mABs as discussed below.

Finally, other factors can induce changes in mAB pharmacokinetics (**Table 2**). For example, vedolizumab clearance increased with body weight values higher than 120 kg (Rosario et al., 2015). Body weight also affects ustekinumab clearance, for which the sex and race of patients were additional factors (Lamb and Duggan, 2017). However, the relationship between infliximab clearance and body weight may vary over the induction and maintenance phases. In particular, non-obese patients could be underdosed during the induction phase comparing to obese patients (Dotan et al., 2014), but the latter tend to clear infliximab more rapidly during maintenance, as it was observed with adalimumab (Billiet et al., 2016).

PERSONALIZED MEDICINE IN INFLAMMATORY BOWEL DISEASES

Both drug monitoring protocols and pharmacogenetic analyses are valuable in exploiting the therapeutic effect of mABs while sparing patients from toxicities. Of note, these endpoints may be combined with clinical markers of efficacy and tolerability (i.e., age, disease severity, and extension) or markers of inflammation (FCP and CRP) to increase the predictive performance of the phenotypic and/or genetic signature (**Figure 1**).

Therapeutic Drug Monitoring

TDM protocols for mABs significantly improve the management of IBD patients in comparison with empiric dose changes based on signs, symptoms, and laboratory results. Low minimum plasma concentrations suggest a dose increase, whereas values within the therapeutic range in association with reduced clinical response indicate a change of drug is needed, as it occurs in the presence of high ADA concentrations (Bendtzen et al., 2009). Furthermore, the optimization of mABs dose does not require a short turnaround time of the test because the long half-life (10–25 days) allows for blood collections two or three days before the next injection, a time that is compatible with the routine execution of the test.

In IBD patients, the therapeutic range of each mAB depends on the severity of the disease and on the endpoint chosen to guide therapy (i.e., clinical or endoscopic remission or normalization of some laboratory findings). It is worth noting that some points deserve a discussion: (1) homogeneity of therapeutic ranges; (2) proactive and reactive TDM; (3) the appropriateness of TMD; (4) the most suitable instrumental platform to quantitate serum concentrations of mABs; (5) the turnaround time of TDM, and finally (6) the strategies that combine TDM of mABs, immunomodulatory drugs, and pharmacogenetic analyses.

Therapeutic Ranges

The identification of a therapeutic range for a drug takes place in clinical trials aimed at establishing a relationship between the administered dose, plasma concentrations, clinical response, and toxic effects. In IBD, the severity of the disease may affect this relationship because a suboptimal clinical or endoscopic/histologic outcome could be related to the most severe disease rather than lowest trough values. Furthermore, therapeutic ranges should reflect the different phases of treatment (induction, postinduction, and maintenance phase) for better control of the active disease in the first phase. A mild albeit therapeutic effect is required in the maintenance phase to keep the disease under pharmacological control for the longest time in the absence of toxic effects.

It is worth noting that the lower bounds of therapeutic ranges also reflect the population considered. As reported above, the recommended trough plasma values of infliximab are higher in children compared with adult patients. Indeed, in 35 children with IBD (23 with CD and 12 with UC), high infliximab C_{min} values (median 6.0 mg/L, range 3.2–12.0 mg/L) during maintenance correlated with combined clinical/biological remission at week 52, whereas low trough concentrations did not (median 2.6 mg/L, range 1.1–3.2 mg/L) (van Hoeve et al., 2019). More recently, another study demonstrated that the percentage of patients with subtherapeutic trough levels (i.e., <5.4 mg/L) at week 14 was higher in young children (<10 years old) than in older ones (≥10 years old) (Jongsma et al., 2020). Importantly, underdosed children required dose increases and developed ADA more frequently than older patients.

Finally, the studies may adopt different endpoints, which include plasma threshold values for clinical remission, mucosal healing, histologic/endoscopic remission, normalization of laboratory exams, and biomarkers (FCP). All of these considerations should also take into account a possible variability between the instrumental platforms and the manufactured tests used for the measurement of mABs and ADA concentrations in plasma (as discussed below). Therefore, many factors can influence the identification of a target therapeutic range, lastly including patient's compliance (Gibson et al., 2020).

Reactive and Proactive Therapeutic Drug Monitoring

Overall, reactive (Papamichael and Cheifetz, 2016) and proactive (Mitrev et al., 2017) approaches for mAB TDM are aimed at comparing drug concentrations and the eventual presence of ADAs to target therapeutic ranges and guide dose optimization. The striking difference between the two is that reactive TDM is suitable when patients under therapy have an unsatisfactorily clinical response, and it can be helpful in the management of secondary loss of response. On the contrary, the proactive approach is appropriate for patients with risk factors for treatment failure (for example, most severe disease and previous anti-TNFα therapies) or most severe consequences after the loss of response (i.e., need for surgery).

The proactive approach has some advantages compared with reactive protocols (Papamichael et al., 2019c) because it is associated with better outcomes, as demonstrated for infliximab (Papamichael et al., 2018) and adalimumab (Papamichael et al., 2019b). Notably, a study found that infliximab trough levels decreased during the first year of treatment; hence, the researchers suggested that "close monitoring of the IFX-TLs (trough levels) could be recommended during maintenance IFX treatment even for patients in remission to be more alert and act a priori" (Orfanoudaki et al., 2019). Furthermore, a prospective Australian study evaluated the impact of TDM on the prescription of infliximab in a real-world setting (Wu et al., 2019), and it found that TDM helped to identify avoidable infliximab dosing with a cost saving. Interestingly, the inappropriate administration was more frequent in reactive (38.9%) than in proactive TDM (19.3%). Data regarding TDM in pediatric studies are scarce (Aardoom et al., 2019), but the adoption of a proactive TDM could be helpful also in children, for whom "IBD is extensive, safety is paramount, and experience with newer biologics is limited" (Carman et al., 2018). For these reasons, proactive TDM is gaining more attention to keep disease under pharmacological control, to prevent loss of response, and to avoid toxicities (Penagini et al., 2020; Vermeire et al., 2020). Interestingly, a third study demonstrated that the incidence of infliximab discontinuation, drug trough levels, and the presence of ADA did not differ between the proactive TDM and the empiric dosing when associated with immunomodulation (Lega et al., 2019).

On the other hand, two systematic reviews and meta-analyses compared empiric dose adjustment and reactive and proactive TDM (Ricciuto et al., 2018; Shah et al., 2020). The first study failed to demonstrate a full advantage for TDM in comparison to the empiric approach in terms of remission rates (Ricciuto et al., 2018). The only significant improvement

resided in a cost-saving effect for reactive TDM and a long therapeutic benefit to anti-TNFα drugs for proactive TDM. In partial agreement with these results, the second study found that only proactive TDM gave some advantages against both the empiric approach (RR, 0.60, 95%CI 0.35–1.04) and reactive TDM (RR, 0.22, 95%CI 0.15–0.22). However, as stated by the authors, the analyses included studies that were heterogeneous for many characteristics, with "very low quality of evidence, mainly due to risk of bias, inconsistency, and imprecision" (Shah et al., 2020).

Overall, TDM is superior to empiric dose adjustment for anti-TNFα drugs, and the reactive approach is a standard of care for IBD patients. More recently, some data are suggesting a propensity of proactive TDM to exploit all of the therapeutic benefits of anti-TNFα mABs (see next section), but the proactive approach is not unanimously considered a standard of care.

Timing of Monoclonal Antibody Therapeutic Drug Monitoring

A panel of experts recently agreed on the timing of TDM for anti-TNFα drugs in IBD patients (Papamichael et al., 2019a). Indeed, proactive TDM is appropriate at the end of the induction phase and at least once in the maintenance period. Patients with primary suboptimal or lack of response and with secondary loss of response would receive reactive TDM (as already discussed in the previous section). For other biologic agents used in IBD (i.e., vedolizumab and ustekinumab), TDM may be considered appropriate at the end of the induction phase (proactive) and when a secondary loss of response is demonstrated (reactive) despite the fact that evidence concerning these drugs is limited (Papamichael et al., 2019a).

Instrumental Platform for Monoclonal Antibody Therapeutic Drug Monitoring

The immunoassays (i.e., ELISA) represent the laboratory reference methods for mAB TDM; thanks to their compatibility with widely diffused platforms already in use in clinical biochemistry laboratories. These characteristics ensure that the majority of IBD patients could benefit from TDM protocols. Different immunoassays for the measurement of plasma concentrations of infliximab and its corresponding ADAs were interchangeable because the findings were significantly correlated (Marini et al., 2017; Nasser et al., 2018), hence strengthening the reliability of TDM from the perspectives of patients and caregivers. Furthermore, based on the available evidence, the variability among tests for infliximab and its ADAs was not clinically significant while the tests did not return different results for infliximab and its biosimilars (Papamichael et al., 2019a). The last finding deserves attention because of the broader use of infliximab biosimilars, even in children (Jongsma et al., 2017).

Although the demonstrated performance of immunoassays, systematic bias, suboptimal specificity, and lack of standardization may characterize the different ELISA methods (Schmitz et al., 2016; van Bezooijen et al., 2016) with inconsistent threshold values of mABs across the studies. The sensitivity and drug tolerance of the available ADA assays represent additional concerns (Egging et al., 2018; Nice et al., 2020). For these reasons, researchers have been engaged in the elaboration and validation of TDM protocols based on liquid chromatography coupled with mass spectrometry (LC-MS). The development of such methods is sometimes laborious (An et al., 2014), the most problematic step being the preparation of peptides by enzymatic digestion (Mouchahoir and Schiel, 2018). Indeed, validated methods often require high expertise in sample preparation and instrumental analysis (Chiu et al., 2018; Willeman et al., 2019), characteristics that may impede the adoption of methods among laboratories.

However, the validation of LC-MS methods for infliximab, adalimumab (Jourdil et al., 2018; El Amrani et al., 2019), and vedolizumab (Schulze et al., 2018) returned findings significantly correlated with those obtained with ELISA immunoassays. Interestingly, the application of an LC-MS method to infliximab TDM gave a plasma concentration threshold of 6.2 mg/L for biological remission (plasma CRP<5 mg/L and FCP<150 μg/g stools) of the disease (Nemoz et al., 2019), a value that was in the lower band of the therapeutic range suggested for mucosal healing in the maintenance period (Ungar et al., 2016; Yarur et al., 2017). In another trial, an LC-MS method did monitor trough levels of vedolizumab in IBD patients (Schulze et al., 2018). Responders had mean values (38.3 and 41.8 mg/L) higher than those of patients with loss of response (33.4 and 39.3 mg/L) in the induction period (weeks two and six of treatment, respectively).

Therapeutic Drug Monitoring of Thiopurines

As stated above, the pharmacological management of IBD may require the administration of thiopurines. However, a correlation may exist between combined treatments and an increased risk for infections and neoplasms (Bots et al., 2018). A solution to reduce those unfavourable outcomes is to administer immunomodulatory drugs for a limited time and under TDM supervision when possible. Indeed, 6-TGN levels >105–125 pmol/8x10^8 erythrocytes were required to reduce infliximab immunogenicity and, hence, ADA production (Colombel et al., 2010; Yarur et al., 2015). Similar findings were obtained for adalimumab when 6-TGN concentrations were ≥223 pmol/8x10^8 erythrocytes (sensitivity 100% and specificity 60.6%) (Nakase et al., 2017). In the case of vedolizumab, concomitant immunosuppressive therapy decreased the formation of ADAs (Feagan et al., 2013; Sandborn et al., 2013). On the contrary, immunomodulation did not affect ustekinumab clearance (Lamb and Duggan, 2017). Therefore, although the modulating effect of thiopurines could be generalized in all patients, their use in combination with mABs may be considered on a case-by-case basis due to the risk of increased adverse reactions.

Pharmacogenes in Inflammatory Bowel Diseases

The evaluation of genetic markers associated with treatment efficacy or tolerability is relevant for both chemical and biologic drugs.

In 2014, Bank and coworkers found that 19 out of 39 functional polymorphisms in 26 genes belonging to the NFκB-mediate inflammatory response predicted the response to anti-TNFα therapy in a large cohort of 482 CD and 256 UC patients (Bank et al., 2014). In 2016, a meta-analysis found that polymorphisms on TLR2, TLR4, TLR9, TNFRSF1A, IFNγ, IL6, and IL1β genes correlated with treatment response in IBD (Bek et al., 2016). Additionally, another polymorphism on the FCGR3A gene was predictive of therapy response in CD patients.

Notably, in 2014, Bank and colleagues replicated the gene signature in a subsequent cohort of 587 CD and 458 UC patients (Bank et al., 2019). The updated signature included ten polymorphisms belonging to NFκB-, TNFα-, and cytokine-signalling pathways. Interestingly, patients with risk signatures for TNFα-driven inflammatory status were most likely prone to experience a benefit from anti-TNFα agents. A subsequent study partially confirmed those findings in 103 IBD patients (80 CD and 23 UC) (Romero-Cara et al., 2018). In particular, a correlation emerged between the wild-type allele of the polymorphism rs396991 (V158F) in the *FCGR3A* gene and the development of ADAs (highest plasma concentration in VV homozygous patients) and reduced concentrations of infliximab. Overall, the studies well stressed the need to investigate multiple causative factors to obtain a genetic signature with good predictive/prognostic performance.

The genetic analyses may pair with clinical or laboratory findings. For example, in 29 CD and 18 UC patients, a study detected a significant correlation between the polymorphism rs1143634 within the promoter region of the IL1β gene, the higher cytokine concentrations at baseline, and reduced response (in terms of clinical remission) to infliximab at week 14 (Lacruz-Guzmán et al., 2013). Similarly, the polymorphism rs2228273 in ZNF133, with thiopurine use and body weight, predicted unsatisfactory response to infliximab after the first administration (Jung et al., 2019).

In recent years, pharmacogenetic analyses have taken advantage of the employ of unsupervised techniques, as well as microarrays, GWAS, and next-generation sequencing platforms, which enable the screening of numerous possible genetic markers at the same time (Di Paolo et al., 2019). In some cases, genetic markers were combined with clinical and laboratory characteristics to obtain highly performant predictive models (Dubinsky et al., 2010). For example, in 231 UC patients of Caucasian ancestry, a recent study found two gene signatures of 8 and 12 SNPs associated with primary non-response (PNR) and duration of response (DR) to anti-TNFα therapies, respectively (Burke et al., 2018). Intriguingly, "genetic risk scores for PNR and DR were not associated with infliximab levels or antibody formation" meaning that "the associations of these SNPs may be mediated by mechanisms other than drug pharmacokinetics or antibody formation." In UC patients, another study evaluated whether a genetic signature developed for infliximab could also predict mucosal healing, clinical response, and remission after treatment with golimumab (Telesco et al., 2018). The findings demonstrated that the signature was mainly drug-specific because it could not identify patients who did achieve clinical remission or response after golimumab treatment. The study also evaluated a possible companion diagnostic for anti-TNFα agents (Kaneider and Kaser, 2018). In 474 IBD patients of European ancestry, two genetic variants, rs116724455 in TNFS4/18 and rs2228416 in PLIN2, were predictive of refractoriness to therapy and increased the predictability of a clinical-based risk model (Wang et al., 2019). Finally, a Spanish research group found a significant association between five polymorphisms in TNFα or NFκB pathways and plasma concentrations of both infliximab (rs5030728 in TLR4 and rs11465996 in LY96) and adalimumab (rs1816702 in TLR2, rs2569190 in CD14, and rs3397 in TNFRS1B) in 154 children affected by IBD (Salvador-Martín et al., 2020) although different regimens and the number of patients in subgroups partly weakened those associations.

The genetic signature discovered by GWAS may be partially disease-specific, as in the study by Jostins et al. (Jostins et al., 2012) who revised 15 GWASs and found that 110 IBD loci were shared between CD and UC, whereas 30 and 23 loci were specific for CD and UC, respectively.

Finally, a very recent GWAS performed in 1,240 biologic-naïve patients found a significant association between the HLA-DQA1*05 locus and both an increased rate of immunogenicity (OR, 1.90) and the development of ADAs against infliximab and adalimumab (Sazonovs et al., 2020). Of note, the use of the biologics alone or in combination with other drugs did not affect this relationship. In another study that enrolled 252 IBD patients, the variant allele of the HLA-DQA1*05 locus significantly increased the risk for ADAs against infliximab (HR = 7.29) independently from age, sex, weight, and immunomodulators (Wilson et al., 2020), which are known factors affecting the elimination of mABs.

DISCUSSION

Tailoring pharmacological therapy for every IBD patient means choosing an effective therapeutic regimen that could be promptly modified in the presence of poor responsiveness to the disease or after the onset of toxic effects. Additional reasons may sustain treatment optimization on an individual basis, as well as the chronic administration of drugs even in combination, their low therapeutic index, and the progressive worsening of the disease. According to these factors, four sequential steps may increase the therapeutic potential of mABs by exploiting their pharmacodynamic and pharmacokinetic characteristics (**Figure 1**).

First of all, pharmacogenomic tests may score the patient's risk of unsatisfactory response to mABs at the time of diagnosis, well before the patient will be considered a candidate to receive a biologic agent (Wang et al., 2019). Indeed, germinal genetic variants may be predictive biomarkers of response to mABs, as done to define the risk of developing CD or UC in different populations (Venkataraman and Rivas, 2019). It is worth noting that in many cases the prediction can be improved by a genetic signature rather than using a single genetic locus.

This strategy may also evaluate the risk of disappointing efficacy or toxicities associated with thiopurines, methotrexate, aminosalicylates, and immunosuppressants (Voskuil et al., 2019), both in adults (Heap et al., 2014; Heap et al.,2016; Kakuta et al., 2018; Walker et al., 2019) and children (Lucafò et al., 2019). This approach is valuable when TDM protocols require chromatographic methods that can be laborious and need specific expertise as in the case of thiopurines. Moreover, pharmacogenomic analyses may predict the tolerability of combined treatment regimens, which are advantageous as second-line therapies (Roblin et al., 2020). For example, a Dutch study did define a genetic passport that included several loci (TPMT, NUDT15, HLA-DQA1*02:01-HLA-DRB1*07:01, and HLA-DQA1*05) associated with toxic effects from thiopurines (i.e., myelosuppression and pancreatitis) and anti-TNFα mABs (i.e., immunogenicity) (Bangma et al., 2020). The signature may work even in combination with clinical risk factors (i.e., previous anti-TNFα therapies) (Rosario et al., 2017), assuming that it is cost-effective in terms of both additional costs for healthcare systems and patients' quality of life (Sluiter et al., 2019). However, the presence of rare variant alleles could weaken that relationship (Zimdahl Kahlin et al., 2019), hence justifying "the use of a combination of genotyping and phenotyping in order to detect as many individuals as possible who are at risk of treatment failures and adverse reactions during thiopurine treatment".

According to that suggestion, the second step considers proactive TDM to better define the responsiveness status during or at the end of the induction phase for responders and nonresponders to anti-TNFα agents, as well as for nonresponders to vedolizumab and ustekinumab (Papamichael et al., 2019a). This approach is mainly valuable for carriers of known risk factors, and this is advantageous in comparison with following empiric dose adjustment or reactive TDM based on signs and symptoms of the disease (Ricciuto et al., 2018; Shah et al., 2020). Although characterized by some drawbacks, immunoassays represent the methods with the broadest diffusion across laboratories.

The third step involves TDM during the maintenance phase when the measurement of mABs and ADAs may be appropriate for all anti-TNFα agents (Papamichael et al., 2019a), while the reactive TDM can be the standard of care for all IBD patients who experienced a loss of response. As presented in previous paragraphs, the titration of ADAs (rather than the notification of their presence or absence) could further improve the segmentation of patients according to their likelihood of response (Brandse et al., 2017; Okamoto et al., 2020).

It is worth noting that the TDM protocols in the second and third steps may take advantage of specific proactive activities, as well as dose optimization according to algorithms that may represent the fourth step. Indeed, *in silico* studies demonstrated that adaptive dosing strategies were superior to stepwise or proportional dosing approaches (Wojciechowski et al., 2017) and two clinical trials evaluated Bayes models (Strik et al., 2019; Santacana Juncosa et al., 2020). In particular, the work of Santacana Juncosa and colleagues demonstrated that the regimen individualization according to a Bayes strategy may significantly increase the percentage of patients in clinical remission and the remission rate for those who need an intensified dosage. Moreover, the disease control lasted in the deintensified cohort, and 92.4% of patients still received infliximab after one year of treatment (Santacana Juncosa et al., 2020).

In conclusion, a combined pharmacokinetic/pharmacogenetic approach may achieve new goals in the treatment of IBD patients; thanks to a tailored approach based on TDM (including Bayes strategies), pharmacogenomic analyses, and clinical records. Likely, adding pharmacodynamic markers (i.e., plasma, cellular, or tissue levels of cytokines as TNFα and IL8) may increase the predictive value of models and, ultimately, the control of the disease with significant improvements in patients' health status and quality of life.

AUTHOR CONTRIBUTIONS

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

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Contribution of Janus-Kinase/Signal Transduction Activator of Transcription Pathway in the Pathogenesis of Vasculitis: A Possible Treatment Target in the Upcoming Future

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Janus-kinase (JAK) and signal transduction activator of transcription (STAT) signal transduction pathway is involved in a wide range of physiological and pathological processes, including in the pathogenesis of several autoimmune diseases. Data supporting the role of JAK/STAT in the development of vasculitis are limited and mostly focused on large vessel vasculitis and Behçet's disease. In this review, we provide a thorough picture of currently available evidence on the topic, gathered from *in vitro* experiments, animal models and human real-life data, analyzing the rationale for the use of JAK inhibitors for the management of vasculitis. Overall, despite a very strong biological and pathogenic basis, data are too few to recommend this therapeutic approach, beyond very severe and refractory forms of vasculitis. However, for the same reasons, a strong scientific effort in this direction is indeed worthwhile.

Keywords: JAK/STAT, vasculitis, giant cell arteritis, Takayasu arteritis, Behçet's disease, JAK inhibitors

INTRODUCTION

Vasculitides are a heterogeneous group of systemic inflammatory diseases characterized by the inflammation of the wall of blood vessels. The etiology is mostly unknown and pathogenesis still only partially understood. They are usually classified on the basis of the vessel dimension into large, medium and small vessel vasculitides, but they also differ in terms of epidemiology, clinical picture, prognosis and treatment (Watts and Robson, 2018).

Janus-kinase (JAK) and signal transduction activator of transcription (STAT) are the main players of a transduction pathway named JAK/STAT, involved in a wide range of physiological and pathological processes. JAKs are phosphotranspherases, i.e., enzymes able to transfer a phosphate residue from adenosine-tri-phosphate (ATP) to another substrate. JAKs do not have any receptorial function and are bound to various cytokine receptors. Upon ligand binding to their membrane receptors, JAKs are activated, and phosphorylate STATs to form a phosphorylated (*p*)-STAT dimer that is capable of migrating into the nucleus and inducing DNA transcription.

Four JAKs and seven STATs have currently been identified, named JAK1, JAK2, JAK3, tyrosine kinase (TYK)2 and STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6, respectively. The specificity of signal transduction is determined by the numerous combinations among the isoforms of the three main players of JAK/STAT pathway, the receptor, JAK and STAT.

JAK/STAT is only one of the several pathways involved in intracellular signal transduction. In fact, some of the most well known cytokines involved in the pathogenesis of autoimmune and inflammatory diseases do not signal through JAKs, including tumor necrosis factor (TNF)α and interleukin (IL)-17. However, STATs are indeed involved in their transcription modulation.

On the contrary, all type I and type II cytokine receptors signal through JAK/STAT pathway. It is well beyond the purpose of this review to list and analyze the role of all these mediators. However, in order to provide a general but clear idea of how extensive the activity of JAK/STAT pathway is, it is necessary to mention the most well known pro- and anti-inflammatory mediators (IL-2, IL-6, IL-21, IL-12, IL-35, interferon—IFN-α, IFN-γ, IL-22, IL-10) and growth factors, such as erythropoietin, granulocyte-colony stimulating factor, granulocyte-monocyte-colony stimulating factor, that signal through it.

It is therefore quite intuitive how important this pathway is for hemopoiesis and host defense. Unsurprisingly, loss-of-function mutations of JAKs and STATs have been associated to a wide spectrum of immunodeficiencies, ranging from severe combined immunodeficiency (SCID) to milder forms of impaired immune responses. On the contrary, gain-of-function mutations have been associated with the development of autoimmunity (rheumatoid arthritis–RA, systemic lupus erythematosus–SLE, etc.) and hematologic malignancies.

Presently, commercially available JAK inhibitors have been approved for the treatment of RA, psoriatic arthritis (PsA) and ulcerative colitis (UC), with numerous preliminary data showing a potential good efficacy in other autoimmune conditions, including SLE.

One of the main features differentiating the various JAK inhibitors is their selectivity for the JAK subtypes. Currently available molecules are not selective for one specific isoform, though show a variable grade of affinity with a preferential binding to one or more subtypes. This aspect may represent an advantage, because, by inhibiting multiple isoforms, more cytokine signals are blocked. However, lack of selectivity is one of the main causes of the most frequent adverse reactions, such as cytopenia as a consequence of the inhibition of the JAK2 isoform. Numerous selective JAK inhibitors are currently under investigation, although it is still not clear whether they will provide a real advantage for the management of inflammatory diseases (Schwartz et al., 2016).

To our knowledge, this is the first published review presenting all currently available data on the potential benefit of the use of JAK inhibitors for the treatment of vasculitides. This area is still largely unexplored. However, in consideration of the great burden severe and unresponsive forms of vasculitis represent in terms of survival and quality of life, the exploration of innovative treatment strategies is of utmost importance.

JANUS-KINASE/SIGNAL TRANSDUCTION ACTIVATOR OF TRANSCRIPTION PATHWAY IN THE PATHOGENESIS OF VASCULITIS

As mentioned above, JAK/STAT pathway activation is tightly linked to the production of multiple cytokines, including type I and II IFN and thus they are involved in the pathogenesis of numerous autoimmune diseases, including vasculitis. Scientific data on the topic are very scarce and the few studies available are mostly focused on giant cell arteritis (GCA), Takayasu arteritis (TKA) and Behçet's disease (BD). A graphical summary of the current evidence on the role of JAK/STAT pathway in the pathogenesis of vasculitis is provided in **Figure 1**.

GCA and TKA are the two most prevalent forms of large vessel vasculitis, preferentially involving the aorta and its larger branches. TKA is more common in young women, mostly in the second and third decade of life, while GCA typically affects people aged 50 years or older (Koster and Warrington, 2017; Watanabe et al., 2020). Differently, BD is a multisystemic chronic inflammatory disease, potentially involving small, medium and large vessels. It typically affects young adults aged 20–40 years and is more prevalent in countries along the ancient silk route.

Despite their etiology remains elusive, there is strong evidence supporting one or more antigens as the triggering factors at the basis of a dysregulated immune response and a consequent self-sustaining chronic inflammation in the context of a genetic predisposition (Carmona et al., 2015; Carmona et al., 2017; Hatemi et al., 2018; Zhang et al., 2018; Régnier et al., 2020).

Despite these forms of vasculitis share common features, such as vessel wall infiltration by immune cells, intimal hyperplasia, aneurysm formation, dissection and stenosis, the pathogenic process and cellular subsets involved are distinct. While T cells represent the key players in sustaining chronic inflammation in GCA and TKA, an aberrant response of the innate immune system is of utmost importance in the pathogenesis of BD (Dasgupta et al., 1989; Martinez-Taboada et al., 2001; Weyand and Goronzy, 2003; Weyand and Goronzy, 2013; Wen et al., 2016; Maleszewski et al., 2017a; Koster and Warrington, 2017; Puccetti et al., 2018; Zhang et al., 2018; Watanabe et al., 2020).

Due to its widespread biological functions, JAK/STAT pathway is potentially involved in all the pathogenic phenomena leading to the development of GCA, TKA and BD.

Giant Cell and Takayasu Arteritis

As far as GCA and TKA are concerned, Th1 and Th17 cells represent the main players in the development of the diseases. It is well known that the former are strictly linked to STAT1, STAT2 and STAT4 activity, while the latter to STAT3. It is in fact possible to induce GCA on healthy human artery engrafted on SCID mice by reconstituting them with peripheral blood mononuclear cell (PBMCs) from GCA patients. As expected, pathological analysis of the grafts demonstrated a robust T cell infiltrate. Additionally, in arteritic tissue lesions from the same GCA mouse model, STAT1 and STAT2-dependent target genes are strongly upregulated and there is an increased production of cytokines like IFN-γ, IL-17 and IL-21 (Watanabe et al., 2017; Zhang et al., 2018).

Further supporting these data, a transcriptome analysis of $CD4^+$ and $CD8^+$ T cells performed on a very large cohort of patients with TKA, showed a significantly increased expression of numerous genes closely related to JAK/STAT pathways such as IL-12, IL-17, IL-19, IL-22 and type I and II IFNs. Additionally, a network analysis showed JAK/STAT pathway-involved genes among those with the highest number of connections, further

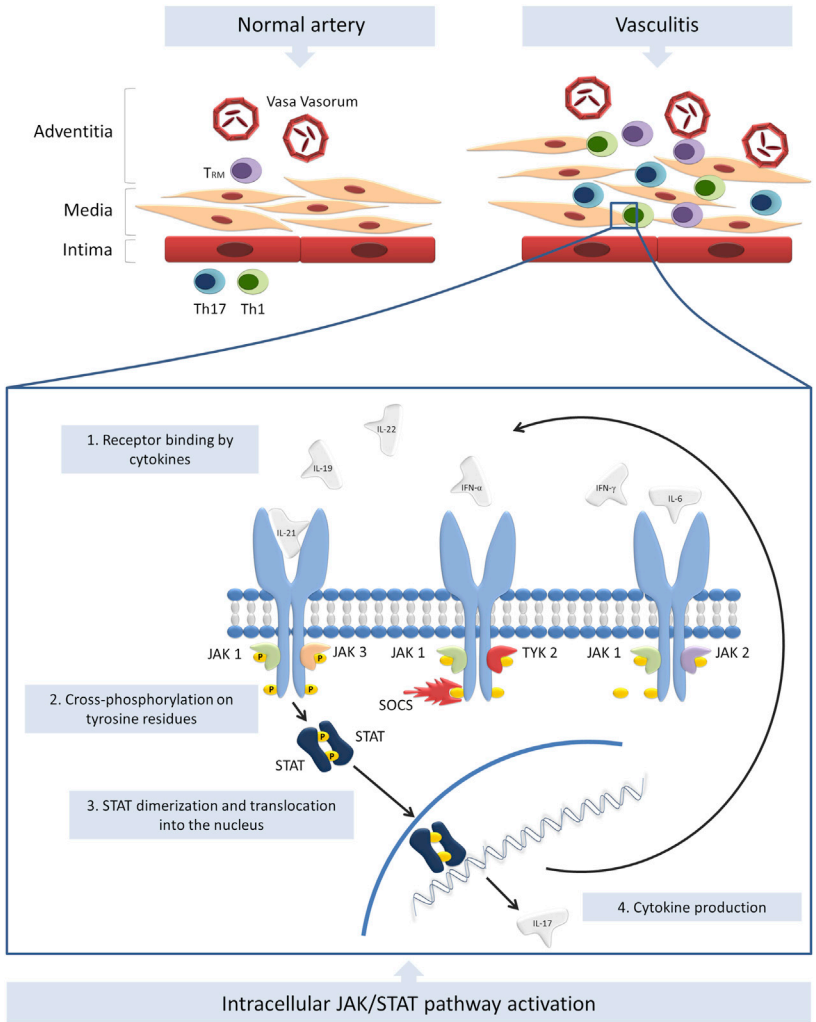

FIGURE 1 | Summary of the current evidence on the role of JAK/STAT pathway in the pathogenesis of vasculitis. T_{RM}, Th1 and Th17 cells infiltrate the vessel wall and induce inflammation. Numerous cytokines are secreted by T cells and innate immunity cells and activate JAK/STAT pathway. Other cytokines, such as IL-17 are therefore produced an secreted, further amplifying the inflammatory response. The modulation of SOCS transcription may be another altered mechanism involved in the pathogenesis of vasculitides.

supporting the hypothesis of their pivotal role in TKA (Weyand et al., 2012; Weyand and Goronzy, 2013; Maleszewski et al., 2017a; Koster and Warrington, 2017; Watanabe et al., 2017; Watanabe et al., 2020; Zhang et al., 2018; Régnier et al., 2020).

Beyond the articular involvement, collagen induce arthritis (CIA) rats, especially if given a high-fat-diet, develop an inflammatory infiltration of the aorta and can thus be employed as models of large vessel vasculitis. The expression of p-STAT3 in the aortic endothelium of CIA rats is significantly increased, suggesting a potential role of JAK/STAT activation in vascular inflammation (Cai et al., 2020).

Noteworthy, a new subtype of no-circulating T memory cells have been recently described in GCA arteritic lesions and named tissue-resident memory T cells (T_{RM}), identified as $CD69^+$ and $CD103^+$. Unlike conventional T cells, T_{RM} do not migrate to secondary lymphatic stations and seem to locally exert a pro-inflammatory effect through the activation of JAK/STAT. Additionally, the presence of infiltrating active T_{RM} has been demonstrated in the vessel wall of grafted temporal arteries in the SCID chimera mice model of GCA. T_{RM} also appear to be implicated in driving long-lasting inflammation through the recruitment of $CD4^+$ lymphocytes (Zhang et al., 2018).

Very few *in vitro* studies have investigated the biology of JAK/STAT activation in T cells from vasculitis patients. However, cultured $CD4^+$ T cells from GCA patients, even in the absence of antigen-presenting cells, have been shown to spontaneously and preferentially differentiate towards a Th1 phenotype and to produce IFN-γ. JAK/STAT pathway proved essential for their survival and activation (Park and Kupper, 2015; Koster and Warrington, 2017; Zhang et al., 2018).

Behçet's Disease

As we described, the pathogenesis of BD is profoundly different from that of GCA and TKA, showing a far greater involvement of the innate immune system. However, T cells and JAK/STAT pathway still seem to play a key part in the development of the disease. In fact, the same pro-inflammatory cytokines (IL-12, IL-17, IL-23, IFN-γ), and T cell subsets (Th1 and Th17) have been shown to be increased in the peripheral blood and serum of BD patients compared to healthy controls (HC) (Puccetti et al., 2018). However, basal levels of STAT3 and p-STAT3 are significantly increased in $CD14^+$ monocytes and $CD4^+$ T cells of BD patients compared to HC. This aspect seems to be in contrast with the prevalent expression of STAT1-2 on STAT3, observed in GCA. Additionally, STAT3 polymorphisms associated to susceptibility to BD have been identified in a Chinese population (Hu et al., 2012; Tulunay et al., 2015; Abdi et al., 2018).

Despite the above-mentioned observations, the role of JAK/STAT pathway in BD is still not clear, with only few and partially conflicting data available in the literature. The great variability of the disease phenotype and its relatively low frequency in most countries probably represent the main reasons of such paucity of data and discrepancies. As an example of conflicting data, an Iranian study found that the gene of suppressor of cytokine signaling (SOCS) 1, a negative regulator of JAK/STAT pathway, was hypermethylated in BD patients compared to HC. This observation would confirm a reduced transcription of SOCS1 itself. However, other authors found that SOCS1 and SOCS3 expression may actually be increased in PBMCs and circulating neutrophils from BD patients (Hamedi et al., 2014; Abdi et al., 2018).

Other Vasculitides

Data on other forms of vasculitis are very scarce and no direct evidence of an involvement of JAK/STAT pathway in their pathogenesis is available. However, some hints of a potential role of JAK/STAT may come from the evidence of an upregulation of STAT3 in the PB $CD4^+$ and $CD8^+$ T cells of polyarteritis nodosa (PAN) patients and of increased levels of CXCL10 (a STAT induced gene) in active Kawasaki disease (Rimar et al., 2016; Berthelot et al., 2020).

JANUS-KINASE INHIBITION AS A STRATEGY FOR THE TREATMENT OF VASCULITIS

Similarly to the data regarding the pathogenic role of JAK/STAT pathway in vasculitis, the evidence of the effects of JAK inhibition is limited.

In Vitro Studies

In vitro inhibition of JAK1/3 on PB-derived $CD4^+$ T cells of GCA patients (which are known to spontaneously differentiate towards a Th1 phenotype) demonstrated a strong reduction of IFN-γ producing cells (Zhang et al., 2018). Similarly, the *in vitro* treatment of TKA patient-derived T cells with the JAK inhibitors ruxolitinib and tofacitinib showed a reduced expression of Th1 and Th17 cells, an increase of $CD4^+$ T regulatory (Treg) cells and a reduction of CD25 expression by $CD4^+$ and $CD8^+$ cells. It has also been hypothesized that the inhibition of JAK1/3 may modulate macrophages and natural killer cells activity (Régnier et al., 2020; Watanabe et al., 2020) and reduce IL-17A-induced activation of human umbilical vein endothelial cells (HUVECs) through the inhibition of p-JAK1, p-JAK2, p-JAK3 and p-STAT3 (Cai et al., 2020).

Animal Models

As briefly mentioned above, JAK/STAT signaling is essential to keep T_{RM} cells alive and functional in order to perpetuate chronic inflammation. Interestingly, despite long-term glucocorticoid treatment, the T cells infiltrating the arterial wall may persist and be responsible of perpetuating inflammation (Maleszewski et al., 2017b). By administering tofacitinib to an engrafted mouse model of GCA, the Authors showed a significant reduction of the T_{RM} population infiltrating the vessel wall, a disruption of their survival signals, a reduction in the expression of lineage-determining transcription factors (T-bet, RORC and BCL6). Consequently, the production of pro-inflammatory cytokines such as IFN-γ, IL-17 and IL-21 was reduced, thus demonstrating a key role of JAK/STAT pathway in this process (Zhang et al., 2018).

As far as the endothelium is concerned, JAK inhibition is able to significantly reduce the tissue levels of growth factors, such as platelet-derived growth factor, fibroblast growth factor 2, vascular endothelial growth factor and, consequently, inflammation-associated microangiogenesis and mio-intimal hyperplasia (Maleszewski et al., 2017a; Koster and Warrington, 2017; Zhang et al., 2018; Cid et al., 2020). Additionally, in CIA rats with aortitis JAK/STAT pathway inhibition is able to restore normal levels of p-STAT3 and to significantly decrease endothelial IL-17A expression, thus reducing the severity of vascular inflammation (Cai et al., 2020).

Clinical Evidence

Real-life data on the efficacy of JAK inhibitors for the treatment of vasculitis are even fewer (mostly coming from case-reports of resistant TKA) and with contrasting results. Albeit one single case-report showed a treatment failure, most published cases demonstrate a significant improvement of refractory disease with a reduction of IL-6 and p-STAT5 serum levels, along with an increase of Treg/T effector cell ratio. Interestingly, tofacitinib appears to be particularly effective in TKA complicated by UC. This may be related to a different underlying genetic background of these subjects (Terao et al., 2015; Abdolahi et al., 2020; Kuwabara et al., 2020; Palermo et al., 2020; Régnier et al., 2020; Sato et al., 2020; Watanabe, 2020; Yamamura et al., 2020).

A possible explanation of the susceptibility of treatment-resistant vasculitis to JAK inhibition may be related to the ability of antigen presenting cells to stimulate Th1 and Th17 (both essential in the pathogenic process) through independent

and distinct signals. Consequently, blocking a single cytokine may not be sufficient to control the disease (Weyand et al., 2012; Weyand and Goronzy, 2013; Cid et al., 2020). Similarly, in PAN different cytokines may stimulate JAK/STAT3 pathway, thus explaining the potential efficacy of JAK inhibitors where the blockade of IL-6 failed. In support of this, a case-report of a long-standing, refractory PAN patient describes a significant response to tofacitinib (Rimar et al., 2016).

Regarding the use of JAK/STAT inhibitors in BD, a pilot study conducted on refractory patients with very heterogeneous disease manifestations and severity showed an overall significant improvement, especially of cardiovascular and joint domains. However, a poor response on gastrointestinal involvement has been observed. Although a clear explanation of this phenomenon is still lacking, it may be important to consider the similarity of BD gastrointestinal involvement with Crohn's disease, in which JAK inhibition is likely not effective (Abdi et al., 2018; Kuwabara et al., 2020; Liu et al., 2020; Sato et al., 2020).

Despite data are very limited, the potential benefit of JAK inhibitors for the treatment of vasculitis may have a significant impact on the management of patients, especially in case of treatment-resistance. For this reason, clinical trials aimed at evaluating the efficacy of JAK inhibitors in GCA (NCT03725202, NCT03026504) and TKA (NCT04161898, NCT04299971) are currently ongoing and preliminary data are expected soon.

DISCUSSION

In the last few years, the role of JAK/STAT pathway in the pathogenesis of several autoimmune diseases has acquired growing attention. Most of the data currently available are focused on RA, PsA and UC, for which the use of JAK inhibitors have been approved. However, due to the involvement of JAK/STAT in the vast majority of the inflammatory processes, it is reasonable to hypothesize a wider use of JAK inhibition, including for the treatment of vasculitis.

Very little data currently support the use of JAK inhibitors in clinical practice. However, the solid biological rationale and a few published case-reports may suggest considering this approach in very severe and refractory forms of vasculitis and represent a strong impulse to persevere on this scientific path.

Nonetheless, because JAK/STAT inhibition carries significant and potentially very severe complications, we are very far from being able to suggest a widespread use of JAK inhibitors in vasculitis.

AUTHOR CONTRIBUTIONS

RB and GC performed literature research. RB, GC, and EB wrote the first draft of the manuscript. RB, GC, and CP conceptualized and designed the picture. All authors contributed to manuscript conception, revision, read, and approved the submitted version.

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Ig Glycosylation in Ulcerative Colitis: It's Time for New Biomarkers

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Background: Ulcerative colitis (UC) is a chronic relapsing disease, which needs a continue monitoring, especially during biological therapies. An increasing number of patients is treated with anti-Tumor Necrosis factor (TNF) drugs, and current research is focalized to identify biomarkers able to monitor the disease and to predict therapeutic outcome.

Methods: We enrolled consecutive UC patients treated with anti-TNF, naïve to biologic drugs. Therapeutic outcome was evaluated after 54 weeks of treatment in terms of clinical remission (Partial Mayo Score -PMS- <2) and mucosal healing (Mayo Endoscopic Score <2). On serum samples collected at baseline and after 54 weeks of treatment, a Lectin-based ELISA assay was performed, and specific glycosylation patterns were evaluated by biotin-labelled lectins. We have also collected 21 healthy controls (NHS) samples, age and sex-matched.

Results: Out of 44 UC patients enrolled, 22 achieved clinical remission and mucosal healing after 54 weeks. At baseline, when Protein A was used as coating, UC patients non-responders showed a reduced reactivity to Jacalin (JAC) in comparison with NHS (p = 0.04). After one year of treatment, a decrease in JAC binding was seen only in responders, in comparison with baseline (p = 0.04). When JAC binding was tested selecting IgG by means of Fab anti-IgG Fab, UC patients displayed an increased reactivity after anti-TNF therapy (p < 0,0001 vs controls). At baseline, PMS inversely correlates with JAC binding when Fab anti-IgG Fab was used in solid phase (r^2 = 0,2211; p = 0,0033). Patients with higher PMS at baseline (PMS ≥5) presented lower binding capacity for JAC in comparison with NHS and with lower PMS patients (p = 0,0135 and p = 0,0089, respectively).

Conclusion: Ig glycosylation was correlated with clinical and endoscopic activity in patients with UC. JAC protein A-selected Ig showed a possible role in predicting therapeutic effectiveness. If these data would be confirmed, Ig glycosylation could be used as biomarker in UC.

Keywords: Ulcerative colitis, IBD, Biomarker, anti-TNF, glycosylation

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INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing disease, characterized by an inflammation affecting the colon and the rectum with superficial mucosal ulceration, rectal bleeding, diarrhea and abdominal pain (Ungaro et al., 2017). Tumor necrosis factor (TNF) plays an important role in UC pathogenesis. Indeed, several immune cells produce high levels of TNF, and this cytokine is known to mediate several pro-inflammatory functions in the inflamed mucosa, promoting even tissue injury (Neurath, 2014). The mechanisms behind the epithelial cell damage are only partially known, but an interesting paper by Nenci et al.(Nenci et al., 2007) showed how TNF induces intestinal cell apoptosis, or, better, "necroptosis", by activating intracellular specific pathways. TNF activates a receptor-interacting protein kinase-1 (RIPK1), and when caspase-8 activity is suppressed, RIPK1 could promote necroptosis by interacting with receptor-interacting protein kinase-3, which regulates the phosphorylation of mixed lineage kinase domain-like protein (MLKL) (Zhou et al., 2020). This translocation of MLKL results to membrane perforation, and subsequently releases damage-associated molecular patterns into extracellular environment, triggering necroptosis (Royce et al., 2019). On this basis, the monoclonal antibodies anti-TNF, like infliximab (IFX) and adalimumab (ADA) were developed for treatment of UC.

Anti-TNF were demonstrated useful in inducing clinical remission, revolutionizing the management of UC in the past two decades, since their use shifted the treatment goals from symptom control to sustained corticosteroid-free remission, and, to a lesser extent, to mucosal healing (Ungaro et al., 2019). However, more than 30% of the UC patients receiving anti-TNF agents do not respond to treatment, and a significant proportion experience a loss of response or intolerance to treatment, even during the first year (Gisbert et al., 2015). An early prediction of therapeutic outcome is one of the most important challenge of clinicians to optimize therapeutic management.

Immunoglobulin (Ig) glycosylation has a strong impact on antibody function, by modifying protein conformation and IgG affinity for Fc-Receptors and complement (Jennewein and Alter, 2017). The conserved N-linked glycosylation site at the asparagine 297 (Asn297) in each of the Cγ2 regions of Fc fragment presents an arboreal glycan structure with two N-acetylglucosamine (GlcNAc) and three mannose (Man) residues, a "core" at which two additional GlcNAc groups are linked, forming the biantennary branches. Galactose (Gal) addition varily occur on each branch, permitting a potential terminal addition of a sialic acid. Only a minority of the chains are terminated by sialic acid (usually α2,6-linked) (Anthony et al., 2008). Fucose residue at the core GlcNAc can be present as well, alternatively substituted by bisecting GlcNAc (Epp et al., 2016). The peculiar position of the structure, near the cavity between the two IgG heavy chains, influences protein stability and Fc effector functions (Yamaguchi et al., 2006; Raju, 2008). Similar glycan residues are present on IgG Fab portions, modifying antigen recognition and binding (van de Bovenkamp et al., 2016).

Numerous immune-relates diseases, such as rheumatoid arthritis, anti-phospholipid syndrome, and ANCA-associated vasculitis among others, present peculiar modifications of glycan residues in circulating Ig (Parekh et al., 1988; Stümer et al., 2017; Culver et al., 2019). In particular, the majority of studies were conducted in rheumatoid artrithis, where anti-TNF treatment can modify peripheral IgG glycan spectrum (Pasek et al., 2006; Croce et al., 2007; Van Beneden et al., 2009). Recently, similar findings were also demonstrated in Inflammatory Bowel disease (IBD) (Trbojevic Akmacic et al., 2015; Simurina et al., 2018), posing the basis for further studies.

Several biomarkers have been proposed in order to evaluate disease activity and predict therapeutic response to anti-TNF in UC patients (Verstockt et al., 2019; Bertani et al., 2020a; Bertani et al., 2020c; Barberio B. et al., 2020; Bertani et al., 2021). However, the role of Ig glycosylation has never been studied with this purpose. To evaluate this hypothesis, we develop a lectin-ELISA assay in order to estimate an affinity spectrum for each lectin and correlate these results with clinical and endoscopic data.

MATERIALS AND METHODS

Patients and Controls

We conducted a retrospective analysis of biological samples of patients with UC collected prospectively at IBD Unit of Pisa University Hospital from March 2018 to March 2020. The diagnosis of UC was previously confirmed by histology. According to statistical power plan, we chose to enroll 44 consecutive patients with UC treated for the first time with anti-TNF (IFX biosimilars CT-P13 or SB2, or ADA originator), divided in 22 responders and 22 non-responders. Therapeutic response was defined in terms of clinical remission (Partial Mayo Score -PMS- <2, without concomitant steroid therapy) and mucosal healing (Mayo Endoscopic Score <2) at week 54. All patients presented a moderate to severe disease activity at baseline (evaluated according to Mayo Score), and underwent a colonoscopy at baseline and after 54 weeks of treatment. We collected age, sex, smoke habits and disease extension at baseline for each patient. Moreover, C-reactive protein (CRP) and fecal calprotectin at baseline and after 54 weeks of treatment were collected as well. In case of non-response, anti-drug antibody development was evaluated by using specific ELISA kit (Theradiag®, Marne-la-Valle'e, France).

A sample of 9 ml of whole blood was drawn at baseline and after 54 weeks of treatment, immediately before drug administration. It was centrifuged at 4000 rpm, aliquoted and frozen at 20°C, according to our standard procedures for every patient treated with biologics at our Unit. Indeed, every patient treated with this drugs is monitored at our Unit with blood tests every drug infusion (in case of intravenous therapies) or every 8 weeks (in case of subcutaneous ones), and 9 ml of blood were

TABLE 1 | Demographic, clinical and serologic characteristics of patients' cohort.

	UC patients (*n* = *44*) *Baseline*	*Follow-up*	
Age (years) (mean and IQR)	31 (24–45)		
Sex (M/F)	19/25		
CRP (mg/dl)	0.65 (0.3–1.38)	0.3 (0.2–0.63)	p = 0.06
Fecal calprotectin (mg/kg)	266.0 (83–464)	126 (50.5–504.0)	p= 0.29
MES	3 (2–3)	2 (1–3)	p = 0.0002
PMS	4 (2–5)	2 (0–5)	*P* = 0.13
AAL (PrA, OD)	0.404 (0.296–0.603)	0.425 (0.297–0.561)	p = 0.19
LCA (PrA, OD)	0.74 (0.579–0.824)	0.741 (0.59–0.886)	p = 0.49
SNA (PrA, OD)	0.587 (0.558–0.63)	0.594 (0.565–0.624)	p = 0.8
JAC (PrA, OD)	0.696 (0.556–0.781)	0.635 (0.536–0.737)	p= 0.07
JAC (fab anti-Fab, OD)	0.252 (0.184–0.5)	0.692 (0.372–0.943)	p < 0.0001

Data are expressed as median (interquartile range–IQR).

CRP *C-reactive protein,* MES *Mayo Endoscopic Score,* PMS *Partial Mayo Score,* AAL *Aleuria Aurantia Lectin,* LCA *Lens Culinaris Agglutinin,* SNA *Sambucus Nigra Lectin,* JAC *Jacalin,* PrA *Protein A,* Fab anti-Fab *Fab anti-IgG Fab,* OD *Optical Density.*

used to create a Biobank of serum samples. Moreover, we have collected 21 healthy controls samples, age and sex-matched.

The present study was approved by the local Ethics Committee, and all patients gave their written informed consent for collection and publication of data.

Lectin-Based ELISA

In order to detect specific glycan structures exposed on IgG, a Lectin-based ELISA assay was performed on the serum samples (Sjöwall et al., 2015; Stümer et al., 2017). Specific glycosylation patterns were evaluated by biotin-labelled lectins: aleuria aurantia lectin (AAL), lens culinaris agglutinin (LCA), O-glycosidically linked galactose/N-acetylgalactosamine (GalNAc) ligand jacalin (JAC) and sambucus nigra lectin (SNA) (Vector Laboratories, United States).

Protein A from *Staphylococcus aureus* (Sigma-Aldrich) was diluted in coating buffer (0.1 M Na_2Co_3/$NaHCO_3$, pH 9,6) to 20 μg/ml and applied onto 96-well MaxiSorpTM microtitre plates (Nunc, Roskilde, Denmark; F96) at 4° overnight (50 μL/well). Alternatively, Fab2-fragment of goat anti-human Fab-specific IgG (Jackson Laboratories Immunoresearch, West Grove, PA, United States) was used at 2 μg/ml.

A blocking buffer (60 μL/well) was then applied onto the plates at room temperature (RT) for 1 h. As blocking buffer, we used 3% deglycosylated gelatin with 0.1% CaCl2 and 0.1% MgCl2. Gelatin was achieved by treatment with periodic acid for 24 h and subsequent dialysis against TBS-Ca-Mg until a pH -value of 7,4 was reached. Sera were diluted 1:500 in washing solution (TBS-Ca-Mg 0.05% Tween-20) and incubated at RT for 3 h (50 μL/well). After 3 washes with washing solution (150 μL/well), plates were incubated at RT for 1 h with biotin-labelled lectins diluted in TBS Ca-Mg (50 mcl/well), at various concentrations (AAL 100 ng/ml, LCA 100 ng/ml, GalNac -Jacalin 50 ng/ml, SNA 50 ng/ml). After washing, HRP-conjugated streptavidin in TBS Ca-Mg was incubated for ½ h at RT (50 μL/well), then washed again and Substrate Solution (TMB) was added (75 μL/well). After 10–15 min stopped with H_2SO_4 (37.5 mcl/well). Optical density values were obtained with the ELISA-reader employing a 450 nm filter. Each serum was tested in duplicate; the final value was obtained by the mean of the two results minus blank. Three samples were used as inter-assay controls. Each test was performed in blind, results were expressed as Optical Density (OD).

Statistical Analysis

The non-parametric Mann–Whitney *U*-test was used to compare the patients' group and the normal subjects. Spearman test was used for correlation analysis. Differences between groups were considered to be significant at a p value of <0.05. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS

Clinical and serological features of the enrolled patients are summarized in **Table 1**. Nineteen out of 44 were male, with a mean age of 31 years. Asthma was the most common comorbidity reported (3 out of 44). Five patients were smokers. Only two patients presented isolated proctitis, while 14 had pancolitis. Before anti-TNF therapy, but not at baseline, seven and 27 patients were treated with topical or oral steroid, respectively (mean dose 15 mg/die prednisone equivalent, SD ± 11). Median CRP level at baseline were 0.65 mg/dl (IQR 0.3–1.38), with 18 patients presenting CRP over normal value (0.5 mg/dl). Median fecal calprotectin was 256 mg/kg (IQR 71–476). Over the 22 patients enrolled as non-responders, seven patients presented anti-drug antibodies at treatment discontinuation (at week 54).

Reactivity for AAL, LCA and SNA showed no differences between healthy controls and UC patients at baseline nor after therapy (**Table 1**). IFX (both biosimilars) and ADA presented negligible lectin binding in comparison with healthy subjects (NHS) and patients (data not shown).

At baseline, when Protein A is used as coating, sera from UC patients non responders to anti-TNF therapy showed a reduced reactivity to JAC in comparison with healthy controls (p = 0.04) (**Figure 1**). After one year of treatment, a modest but significant decrease in JAC binding was seen only in cohort of UC patients responders to therapy, in comparison with baseline (p = 0.04, Wilcoxon paired test).

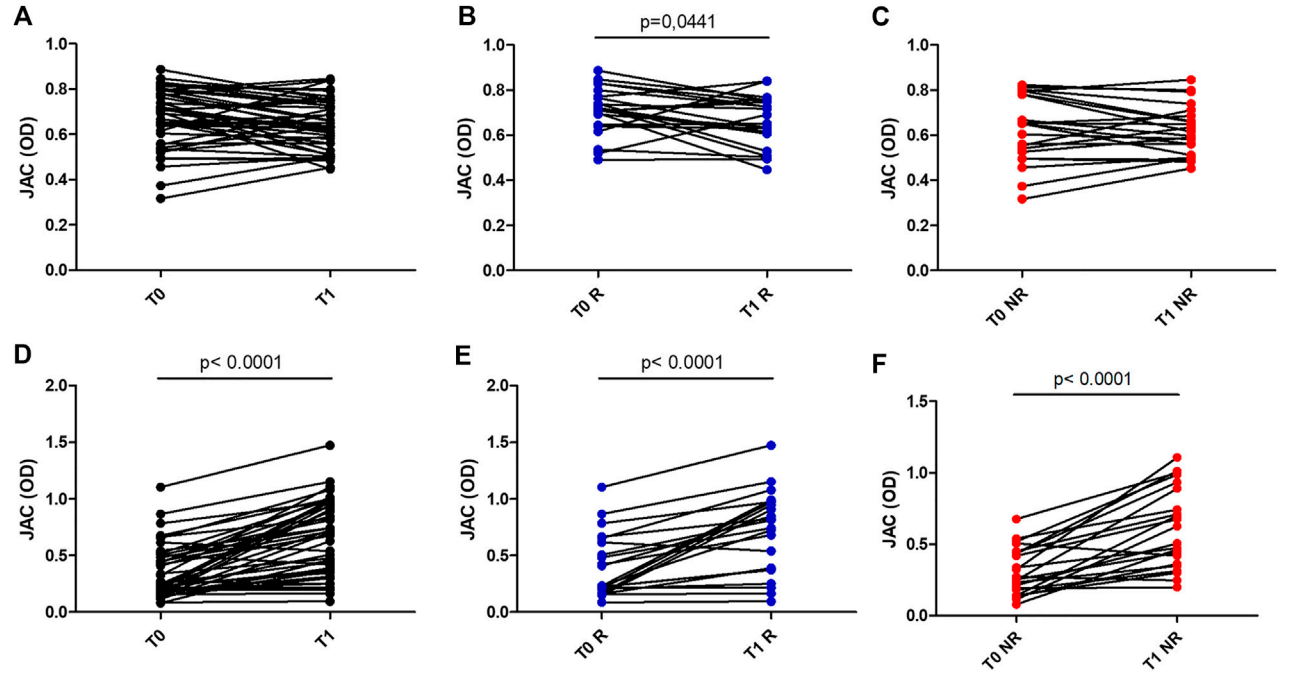

FIGURE 1 | JAC affinity for IgG complex at baseline (T0) and after anti-TNF therapy (T1), expressed as Optical Density (OD). A,B,C: coating with Protein A in all patients **(A)**, in Responders **(B)** and Non Responders **(C)**. D,E,F: coating with Fab anti Fab IgG in all patients **(D)**, in Responders **(E)** and Non Responders **(F)**. All Responders (R) dots were in blue, All Non Responders (NR) dots were in red.

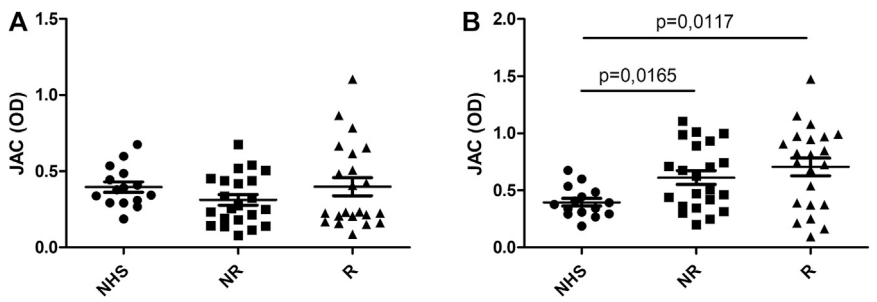

FIGURE 2 | JAC affinity for IgG complex in Ulcerative Colitis compared to healthy subjects (NHS). Lectin-based ELISA coated with Fab anti Fab IgG. **(A)**: at baseline (T0); **(B)**: after anti-TNF therapy (T1). NHS = healthy subjects, R = responders, NR = non responders. All values are expressed as Optical Density (OD).

Conversely, when JAC binding was tested selecting IgG by means of Fab anti-IgG Fab, UC patients display an increased reactivity after anti-TNF therapy (p < 0,0001 vs. controls) (**Figure 2**). Such an increase was higher in responders vs. non-responders (p = 0,0086 and p = 0,0047, respectively).

No correlation was detected with regard for patients' age, sex, smoke habits, steroid use, baseline or follow-up CRP and fecal calprotectin levels (data not shown). No differences were highlighted between patients stratified according to Mayo Endoscopic Score before or after therapy.

At baseline, PMS inversely correlates with JAC binding when Fab anti-IgG Fab is used in solid phase (r^2 = 0,2211; p = 0,0033), while statistical significance was lost when evaluated in follow-up samples (**Figure 3**). Patients with higher PMS at baseline (PMS ≥5) presented lower binding capacity for JAC in comparison with NHS and with lower PMS patients (p = 0,0135 and p = 0,0089, respectively). At week 54, no differences were seen between high PMS and low PMS patients in terms of JAC reactivity (**Figure 3**).

DISCUSSION

The present study showed that Ig glycosylation could be used as biomarker of disease activity in UC, especially before starting a biological therapy with anti-TNF.

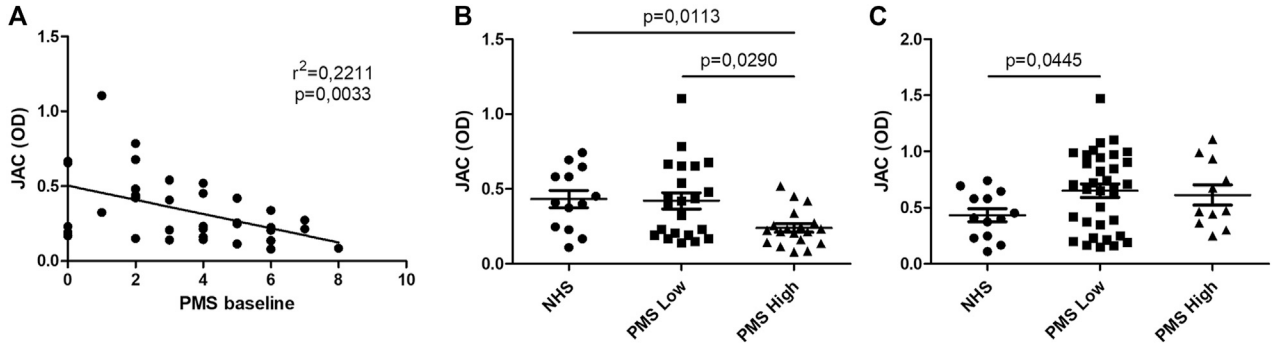

FIGURE 3 | JAC affinity for IgG complex in comparison with Partial Mayo Score (PMS). Lectin-based ELISA coated with Fab anti-IgG Fab. **(A)**: linear correlation between PMS and JAC affinity. **(B, C)**: JAC affinity for IgG complex in Ulcerative Colitis with Low PMS (≤5) and High PMS (>5) compared to healthy subjects (NHS), at baseline **(B)** and after anti-TNF therapy **(C)**. All values are expressed as Optical Density (OD).

UC is a chronic inflammatory disease which needs a continue monitoring. In this perspective, the use of biomarkers is crucial, since they could allow a more detailed evaluation of patients' clinical conditions. Moreover, biomarkers able to reflect endoscopic activity are useful in reducing the number of endoscopic examinations, which are not always well accepted by patients. CRP is one of the most used biomarkers in inflammatory conditions, but its levels are not often correlated to UC activity, unlike Crohn's disease (Saverymuttu et al., 1986). Conversely, fecal calprotectin is the most used biomarker in UC setting (Mumolo et al., 2018): it is reliable both in monitoring disease activity (Mumolo et al., 2018) and in predicting therapeutic effectiveness to anti-TNF drugs (Bertani et al., 2020a), due to its important correlation with endoscopic and histologic activity (D'Amico et al., 2020). However, fecal calprotectin levels could vary day-to-day, and several factors could modify them, such as the use of non-steroidal anti-inflammatory drugs or the presence of other inflammatory conditions (Bertani et al., 2020b). Therefore, several different serum biomarkers have been proposed, and the levels of Leucine-rich α-2 glycoprotein have demonstrated an interesting correlation with tissue inflammation in UC (Serada et al., 2012).

IgG glycosylation represent a post-translational modification capable of fine-tuning the antibody-mediated immune response. Many autoimmune conditions are associated with qualitative perturbations in circulating antibodies. Agalactosylation of N-linked glycans in IgG is considered a marker of inflammatory condition, as shown in patients with rheumatoid arthritis and systemic lupus erythematous (Parekh et al., 1988; Vuckovic et al., 2015). Biantennary agalactosylated antibodies, especially anti-citrullinated protein antibodies, are increased before the disease onset and are capable to interact with FcγRIIa, induce macrophage activation and, subsequently, production of Tumor Necrosis Factor α and IL6 (Clavel et al., 2008).

In their seminal work in 2006, Kaneko and Nimmerjahn demonstrated an anti-inflammatory activity of IgG, as a result of Fc sialylation (Kaneko et al., 2006). This modulation required DC-SIGN receptor interaction (Yabe et al., 2010). Since galactosylation is mandatory for subsequent sialylation, some authors suggested that a lack of galactose induced inflammation reducing sialic immunomodulant activity. Recently, Pfeifle et al.(Pfeifle et al., 2017) demonstrated that IL23 specifically suppress ST6GAL1 expression in plasma cells, inducing a phenotypic switch in Th17 compartment in an IL22/IL23-dependent manner. In turn, ST6GAL1 downregulation in plasma cells lead to asialylated antibody production, shifting toward a pro-inflammatory antibody repertoire (Pfeifle et al., 2017).

A genome-wide association study of IgG N-glycosylation showed that variants affecting the expression of genes involved in the regulation of glycoenzymes colocalize with variants affecting risk for inflammatory diseases: notably, authors observed pleiotropy between variants in the IKZF3-ORMDL3-GSDMB-ZPBP2 locus with inflammatory conditions, including UC (Klaric et al., 2020). An international research group coordinated by prof Lauc clearly described in two recent papers specific modifications in Ig glycome of patients with IBD, analyzed by ultra-performance liquid chromatography (Trbojevic Akmacic et al., 2015) and mass spectrometry (Simurina et al., 2018). IBD patients presented lower levels of IgG galactosylation than controls, while a significant decrease in the proportion of sialylated structures was seen only in patients with Crohn's disease. Notably, a decreased galactosylation was associated with more severe disease for all patients and with a major need for surgery.

Several studies showed that agalactosylation (indicated also as IgG-G0) could be reverted in therapeutic conditions capable of ameliorating the inflammatory diseases. In rheumatoid arthritis, steroid and anti-TNF therapy induce an increase IgG sialylation (Pasek et al., 2006; Croce et al., 2007; Van Beneden et al., 2009). To our knowledge, this is the first attempt to evaluate IgG glycan patterns in UC patients before and after anti-TNF therapy.

We implemented a Lectin-based ELISA, modifying protocols already published (Sjöwall et al., 2015; Stümer et al., 2017). In order to explore IgG glycans, biotin-labelled lectins were used:

AAL, that binds preferentially fucosylated residues; LCA, able to recognize tri-mannose N-glycan core; JAC, an O-glycosidically linked GalNAc ligand; and SNA, capable to bind sialylated residues. This test presents some advantages in comparison with mass spectrometry or capillary electrophoresis: it is affordable, easy to realize in a lab, and did not need denaturation treatments for sample analysis. However, it presents some limits. First, lectins present a gradient of binding capacity for many glycan residues: in this way, a specific lectin reactivity for IgG-complex should be carefully interpreted as the resultant of many different interactions between the lectin and glycan structures, leading to a less specific result in comparison with other technologies. Second, the system is not able to distinguish between Fab and Fc glycosylation, nor can estimate the percentage of biantennary or monoantennary residues, but evaluate IgG-complex as a whole. Moreover, some authors stressed that a lectin-based ELISA could evaluate only IgG complex, e.g., circulating immunocomplex or IgG complexed with acute phase reactants such as CRP, or complement components such as C1q or C3c, since in physiological conditions these complexes are represent verily in peripheral blood (Sjöwall et al., 2015; Stümer et al., 2017). In our cohort of patients, CPR levels did not correlate with lectin binding; on the other hand, an old paper reported the presence of circulating immunocomplexes in UC, albeit clinical implications of this finding were dubious (Kemler and Alpert, 1980). However, a test more adherent to a physiological native IgG condition could be more informative in terms of clinical correlation in comparison with more sophisticated analyses.

Our results diverge when different type of coating was implemented. The putative role of JAC in predicting therapeutic effectiveness was not clearly demonstrated: it could be used as a biomarker of therapeutic response when we evaluated Protein A-selected Ig, whereas in Fab anti-IgG Fab ELISA it was not associated to therapeutic outcome.

The apparent contradiction between data obtained with Protein A or Fab anti-IgG Fab could be explained because of the different orientation of IgG-complexes on the plate. In fact, Fab anti-IgG Fab could expose more clearly the Fc fragment for lectin interaction, or could interfere with immunocomplex formation. Moreover, Protein A (usually used in IgG purification process) could hypothetically recognize with low affinity also circulating IgA. JAC presented high affinity for O-linked GalNAc, which are a minority in human IgG, mainly limited to IgG3 subclass, while classically represented in IgA (Plomp et al., 2015). When IgG selection is more specific, as in the case of Fab anti-IgG Fab, the use of JAC could lead a more informative result. In fact, it has been shown that JAC can recognize as well lactose and galactose, but also mannose and oligomannosides (Bourne et al., 2002). The correlation with a clinical composite score, such as PMS as demonstrated by our results, strongly supports this conclusion. From this point of view, what JAC shows in UC patients after anti-TNF treatment is in line with similar modifications already described in rheumatoid arthritis (Pasek et al., 2006; Croce et al., 2007; Van Beneden et al., 2009; Stümer et al., 2017), suggesting a not disease-specific pathway of anti-TNF in restoring fully glycosylated moiety. Further experiments with mass spectrometry are needed to confirm these findings.

It is worthy to mention that seven patients non-responders developed anti-drug antibodies. This peculiar subgroup did not present a specific glycosylation pattern in comparison with other UC patients. In this perspective, the putative correlation between JAC and clinical activity could be reliable even in case of development of anti-drug antibodies, if confirmed in larger samples.

The present study has some limitations. The retrospective analysis undoubtedly limited the significance of our results. However, it is worth to note that the biological samples and clinical and endoscopic data were collected prospectively, and this procedure limited the possible biases of a typical retrospective study of medical records. Other important limitations are related to the relatively small sample size and to the lack of the evaluation of serum drug levels and anti-drug antibodies in all patients. We should highlight how the present study should be intended as a pilot, explorative study, and we have performed drug levels and antibodies according to the current guidelines (Papamichael et al., 2019), only in case of non-response. A larger cohort would probably increase the significance of our results, in particular with regard for the correlation with endoscopic activity at baseline, where only a trend was showed by our results. Furthermore, larger studies are needed to better characterize the putative role of JAC as a biomarker of therapeutic response to anti-TNF.

Conversely, the present study has an important strength: this is the first time when the role of Ig glycosylation was showed in a cohort of patients with UC all treated with anti-TNF for the first time, highlighting the differences with NHS. Furthermore, the correlation with clinical and endoscopic (although not significant) activity, as well as the behavior during anti-TNF treatment is in line with results obtained with mass spectrometry in patients with IBD and similar to the findings in other immune-related diseases. Therefore, our results pave the way for future studies aimed at clarifying the possible use of Ig glycosylation (particularly JAC), in UC setting.

To conclude, Ig glycosylation may be correlated with clinical and endoscopic activity in patients with UC. Moreover, when a specific Fab anti-IgG Fab was used, JAC affinity increased after anti-TNF therapy and inversely correlated with clinical activity. JAC protein A-selected Ig showed a possible role in evaluating therapeutic effectiveness. Our JAC-labelled ELISA could be an affordable, feasible and reproducible test to evaluate the affinity spectrum of native circulating Ig-complex and it could be a candidate as a new biomarker in UC, if confirmed in larger cohorts.

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 author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CEAVNO. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RC, PM, and LB: Conceptualization and writing of the manuscript; FZ, SM, IP, FC: Clinical data collection; NB, MB, FC, SM: Rewiew and editing of the manuscript. All authors approve the final version of the manuscript.

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frontiers in Pharmacology

REVIEW
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Precision Medicine in Inflammatory Bowel Diseases

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During the last decades, a better understanding of the mechanisms sustaining the pathogenic process in inflammatory bowel diseases (IBD) has contributed to expand the therapeutic armamentarium for patients with these disorders. Alongside with traditional therapies, monoclonal antibodies against tumor necrosis factor-α, the interleukin (IL)-12/IL-23 p40 subunit and the α4β7 integrin, and tofacitinib, a small molecule inhibiting intracellular pathways downstream to cytokine receptors, have entered into the clinic. However, these drugs are not effective in all patients and some responders can lose response over time. Such a therapeutic failure is, at least in part, dependent on the fact that, in IBD, the tissue damage is driven by simultaneous activation of multiple and distinct immune-inflammatory signals and the detrimental mucosal immune response changes over time even in the same patient. Therefore, personalized approaches aimed at identifying which patient should be treated with a specific drug at a precise time point are worth pursuing. A such approach has the advantage to improve efficacy of the drug and limit adverse reactions, thereby improving quality of the life of the patients and reducing costs. In this review, we summarize all the available evidence about the possible role of precision medicine in IBD.

Keywords: crohn's disease, ulcerative colitis, IBD, personalized medicine, anti-TNF

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic, disabling, immune-mediated disorders of the gastrointestinal tract encompassing two main clinical entities: Crohn's disease (CD) and ulcerative colitis (UC) (Abraham and Cho, 2009). Although the aetiology of IBD is unknown, it has been suggested that IBD-associated tissue damage process is induced by an exaggerated immune response against luminal antigens, which is favoured by genetic predisposition and environmental factors (Macdonald and Monteleone, 2005; Digby-Bell et al., 2020).

In the last decades, the possibility to collect mucosal samples from inflamed gut of IBD patients, the use of preclinical models of intestinal inflammation and the advent of sophisticated molecular technologies have led to a better understanding about the mechanisms by which the local immune response promotes gut damage. This progress has promoted the development of many pharmacological compounds, which can target key factors of the IBD-associated mucosal inflammation (Marafini et al., 2019). Among these, various anti-cytokine and anti-integrin blockers and small molecules inhibiting Janus kinases (JAK) are now available for the treatment of IBD (Neurath, 2017; Friedrich et al., 2019; Digby-Bell et al., 2020). However, these drugs are not effective in all patients and some responders can lose response over time. The reasons why blockade of major inflammatory pathways is not beneficial in some IBD patients remain unknown even though a considerable amount of work has been made to

TABLE 1 | Summary of the current evidence on precision medicine in IBD.

Field of investigation	Disease	Class of drug	Summary of evidence	References
Molecular endoscopy	CD	anti-TNF	TNF positive cells detected through endoscopic confocal laser endomicroscopy predict response to anti-TNF	Atreya et al. (2014)
	CD	anti-integrin	α4β7 positive cells detected through endoscopic confocal laser endomicroscopy predict response to vedolizumab	Rath et al. (2017)
Transcriptomics	UC	anti-TNF	Differentially expressed genes separated responders from non-responders to infliximab therapy	Arijs et al. (2009)
	CD, UC	anti-TNF	High levels of oncostatin M in the gut are associated with non-response to anti-TNF therapy	West et al. (2017)
	CD, UC	anti-TNF	The percentage of plasma cells in colon biopsies is a biomarker of failure to anti-TNF	Gaujoux et al. (2019)
	CD, UC	anti-TNF	Up-regulation of CCL7-CCR2 pathway and down-regulation of TREM1 is present in non-responders to anti-TNF	Gaujoux et al. (2019)
	CD, UC	anti-TNF	TREM1 is down-regulated in patients responsive to anti-TNF	Verstockt et al. (2019)
	UC	anti-integrin	Increased mucosal levels of granzyme a and integrin αE are associated with response to etrolizumab	Tew et al. (2016)
Genetics	UC	anti-TNF	Patients homozygous for high-risk IL-23R variants are more likely to respond to infliximab	Jurgens et al. (2010)
	CD	anti-TNF	Fas ligand CC or CT genotype is associated with a higher rate of clinical response to infliximab than the TT genotype	Hlavaty et al. (2005)
	CD	anti-TNF	Homozygous variants of the IBD5 locus are associated to infliximab unresponsiveness	Urcelay et al. (2005)
	Early onset IBD	haematopoietic stem cell transplantation	Mutations in IL-10RA and IL-10RB are associated with a better outcome	Kotlarz et al. (2012)
Immunoprofiling	CD	anti-IL23p19	Baseline serum concentrations of IL-22 predict response to anti-IL-23p19	Sands et al. (2017)
Gut microbiome	CD	anti-integrin	Roseburia inulinivorans and burkholderiales species are more abundant at baseline among patients responders to vedolizumab	Ananthakrishnan et al. (2017)

explain the different outcomes of biologic therapy in IBD. One possibility is that some drugs are effective only in phases of the disease characterized by enhanced production/function of the target, in line with the demonstration that the cytokine response differs among patients and even in the same patient during the disease course (Zorzi et al., 2013; Eftychi et al., 2019). It is, also, conceivable that suppression of a specific inflammatory pathway can, paradoxically, activate additional and distinct immune signals, which amplify the pathogenic process. This occurs, for instance, in patients receiving TNF blockers, in which neutralization of TNF function has been associated with induction of pathogenic T helper (Th)-17 cell responses (Schmitt et al., 2019). Independently of the basic mechanisms underlying such a therapeutic failure, the above observations suggest the need for criteria to stratify patients and to tailor drugs individually. As for now, the therapeutic decision is made by the physician upon critical evaluation of patient's age, disease activity and behaviour, and previous therapies (Ding et al., 2016; Kopylov and Seidman, 2016). Some clinical and demographic characteristics, including age of the patients, smoking habit, penetrating and perianal CD or biochemical parameters (C-reactive protein and albumin), can help guide therapy, but fail to provide information on the preferred class of drugs to select. Therefore, personalized approaches aimed at identifying which patient should be treated with a specific drug at a precise time point are worth pursuing. This would have the advantage to improve efficacy of the drug and limit adverse reactions, thereby improving quality of the life of the patients and reducing costs. Here, we revise all the available evidence about the possible role of precision medicine in IBD (**Table 1**).

MOLECULAR ENDOSCOPY

The use of molecular endoscopy is a revolutionary approach to predict response to therapy in IBD. During colonoscopy, fluorescent antibodies anti-TNF can be topically sprayed directly onto the diseased mucosa and endoscopic confocal laser endomicroscopy facilitates detection and quantification of mTNF-bearing mucosal cells. Atreya and colleagues demonstrated that CD patients with high number of mTNF positive cells in the colon had significantly higher short-term response rates (92%) at week 12 after subsequent anti-TNF therapy as compared to patients with a low number of mTNF positive cells (15%) (Atreya et al., 2014). Moreover, this clinical response was maintained for 1 year of follow-up and was associated with mucosal healing (Atreya et al., 2014). Promising results were also obtained by the same group in a subsequent study, in which, fluorescent antibodies assessing the number of α4β7-positive cells in inflamed gut of CD patients were used to predict response to Vedolizumab, an antibody targeting the α4β7 integrin (Rath et al., 2017). Two patients with pericryptal α4β7-positive cells in inflamed mucosa showed sustained clinical and endoscopic remission to subsequent Vedolizumab therapy, while no α4β7 positive cells were observed during

ex vivo confocal laser endomicroscopy in 3 patients with CD unresponsive to vedolizumab (Rath et al., 2017).

Altogether, these observations suggest that the use of molecular imaging may predict therapeutic responses to biological treatment and can be exploited for precision medicine in CD. Validation in multicentre studies on larger cohorts of patients is needed before this approach can be adopted in clinical practice.

TRANSCRIPTOMICS

In recent years, many studies have been performed to assess whether transcriptomics, the study of gene expression, can predict response to biologics in IBD. Arijs and co-workers compared pre-treatment colonic mucosal gene signature profiles between responders and non-responders to infliximab in a cohort of UC patients refractory to conventional treatment (Arijs et al., 2009). The authors found 212 probe sets differentially expressed between patients who subsequently responded to infliximab and those who did not. The top five differentially expressed genes separated responders from non-responders with 95% sensitivity and 85% specificity (Arijs et al., 2009). West and colleagues showed that high levels of oncostatin M (OSM), its receptor (OSMR) and the related transcriptional modules in inflamed gut of IBD patients were associated with non-response to anti-TNF therapy (West et al., 2017). Moreover, in preclinical models of IBD, genetic deletion or pharmacological blockade of OSM significantly attenuated colitis (West et al., 2017). Overall, these findings support the pathogenic role of OSM in the gut and suggest that unresponsiveness to anti-TNF may be related to the activation of alternative pathways of tissue damage. Gaujoux et al. analysed publicly available genome expression profiles of colon biopsy samples derived from different cohorts of patients with IBD (Gaujoux et al., 2019). The authors found that the percentage of plasma cells was a robust pre-treatment biomarker of failure to anti-TNF therapy. These results were validated in 2 independent cohorts of immune-stained colon biopsy samples, where a plasma cellular score from inflamed biopsies was predictive of non-response. Non-responders to anti-TNF exhibited also up-regulation of CCL7-CCR2 pathway and down-regulation of TREM1 (Gaujoux et al., 2019). However, conflicting results were published by Verstock et al. who found that levels of circulating TREM1 were down-regulated in both CD patients and UC patients responsive to anti-TNF (Verstockt et al., 2019). Factors accounting for such a discrepancy remain unknown even though differences could, at least in part, rely on the definition of responsiveness to anti-TNF adopted in these studies (i.e., clinical response vs. endoscopic response respectively). Transcriptomics were also used to predict therapeutic response to etrolizumab, a monoclonal antibody neutralising the β7 integrin subunit. In UC patients, increased mucosal levels of granzyme A and integrin αE were significantly higher at baseline in patients with subsequent response to etrolizumab (Tew et al., 2016).

GENETICS

More than 200 susceptibility genes have been identified in IBD population (Jostins et al., 2012; Liu et al., 2015; de Lange et al., 2017). Some of these genes have also been studied as possible predictors of response to biologic therapy. For example, patients homozygous for high-risk IL-23R variants were more likely to respond to infliximab therapy compared to patients bearing low-risk IL-23R variants (Jurgens et al., 2010). In a Belgian cohort of 287 consecutive patients treated with infliximab for refractory luminal (n = 204) or fistulizing (n = 83) CD, the Fas ligand −843 CC or CT genotype was associated with a higher rate of clinical response to infliximab than the TT genotype (Hlavaty et al., 2005). Many other loci were found to be predictive of anti-TNF therapy response. For example, the homozygous variants of the IBD5 locus was associated to infliximab unresponsiveness in CD, but not UC, patients (Urcelay et al., 2005).

Many studies have examined whether NOD2, the first and strongest susceptibility gene identified for CD (Cuthbert et al., 2002), is useful to predict response to therapy. Two studies failed to demonstrate a link between NOD2 expression and response to infliximab (Mascheretti et al., 2002; Vermeire et al., 2002). A subsequent metanalysis of 4 studies confirmed that NOD2 polymorphisms were not significantly associated with response to adalimumab or infliximab (Wang et al., 2016). More recently, it was shown that CD patients bearing polymorphisms in NOD2 had anti-TNF trough levels in the subtherapeutic range more frequently than patients without such a polymorphism (Schaffler et al., 2018).

Paediatric patients with very early onset of IBD represent a rare sub-group of IBD that develop the disease early in life due to the presence of monogenic defects (Glocker et al., 2009). In this subgroup, mutations in IL-10RA and IL-10RB were associated with a better outcome after haematopoietic stem cell transplantation (Kotlarz et al., 2012) compared to patients with epithelial gene defects (Uhlig and Muise, 2017).

Inflammasomes are multiprotein complexes of the innate immunity that contribute to the activation of inflammatory response (Sutterwala et al., 2007). Upon stimulation, the inflammasomes promote the maturation of the pro-inflammatory cytokines IL-1β and IL-18 (Opipari and Franchi, 2015). In a patient with a gain of function mutation in NLRC4 (a gene encoding for a protein activating the inflammasome) and developing early enterocolitis, there was an excessive production of IL-18. Notably, treatment of the patient with IL-18 blocker attenuated the ongoing intestinal inflammation (Canna et al., 2017).

These studies highlight the possibility to exploit genetic data to apply personalized therapeutic approaches.

IMMUNOPROFILING

The best example of the use of immunoprofiling to predict therapeutic response is represented by the discovery that baseline serum concentrations of IL-22 in CD predicted response to anti-IL-23p19 (Sands et al., 2017). IL-23 is

produced by various immune cells, especially antigen presenting cells, and is a key cytokine for the maintenance and expansion of Th17 cells, which in turn, together with other cell types, are responsible for IL-22 production (Neurath, 2019). In a phase IIA, placebo-controlled study of 119 adults with moderately-to-severely active CD, patients taking MEDI 2070, an anti-IL-23/p19 antibody, had greater reductions in serum IL-22 levels than did patients receiving placebo. Baseline serum IL-22 concentrations with a median value of less than 15.6 pg/ml were associated with clinical response and remission rates similar to patients receiving placebo, while patients receiving MEDI2070 with levels over this threshold had an increased likelihood of clinical response and clinical remission at week 8 (Sands et al., 2017). Although this study suggests the attractive hypothesis that serum levels of IL-22 can be used as a biomarker to predict response to IL-23p19 inhibitors, larger validating cohorts are required to bring this knowledge into clinical practice.

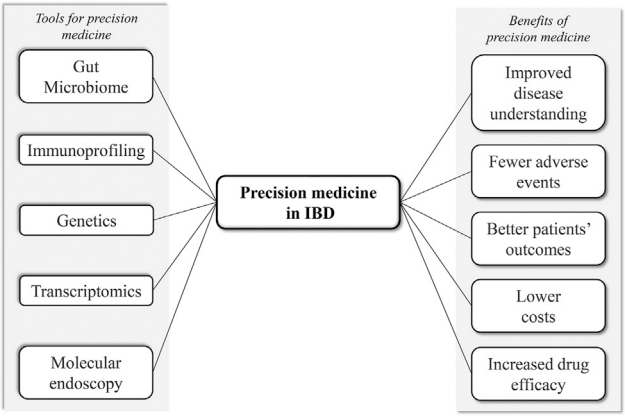

FIGURE 1 | Schematic figure summarizing the tools and possible benefits of precision medicine in IBD.

GUT MICROBIOME

The analysis of gut microbiota is another tool, which can be used to predict response to therapy. Ananthakrishnan and colleagues conducted a prospective study in 85 IBD patients initiating anti-integrin therapy with vedolizumab (Ananthakrishnan et al., 2017). α-diversity was significantly higher among CD patients achieving remission at week 14. Moreover, Roseburia inulinivorans and Burkholderiales species were more abundant at baseline among CD patients achieving remission at week 14. Thirteen pathways were significantly enriched in baseline samples from CD patients achieving remission. No statistically significant differences were observed in UC patients (Ananthakrishnan et al., 2017). These data suggest that microbial changes may be used as promising marker of response to biologic therapies.

DISCUSSION

Considering the continuous enrichment of IBD therapeutic armamentarium, a major challenge is represented by the validation of biomarkers that can be used in clinical practice to predict response to therapy. In fact, clinical trials and real-life studies indicate that response to therapy is highly heterogenous among patients. Thus, the strategy to give the right drug, to the right patient at the right time has become a great research interest in this field. Although individual biomarkers may be promising, the use of a multimodal analysis in which clinical, endoscopic, genetic, transcriptional and immunological data are combined together could build a truly personalized approach. In 2017, Barber and colleagues using a prospective registry, predicted the response of 359 CD patients to their first anti-TNF therapy using clinical and genetic parameters combined together (Barber et al., 2016). In another prospective inception cohort study of paediatric patients with newly diagnosed CD in the United States and Canada, genotypes, ileal gene expression, antimicrobial serology, and ileal, rectal, and faecal microbiota were assessed in order to create a risk model for disease complications and efficacy prediction of subsequent anti-TNF therapy (Kugathasan et al., 2017). This approach allowed a more precise risk stratification and a better selection of patients more likely to benefit from anti-TNF therapy. A similar approach was applied in a cohort study recruiting paediatric patients with newly diagnosed UC. RNA sequencing was used to define rectal gene expression before treatment, and 16S sequencing was used to characterise rectal and faecal microbiota. After adjusting for clinical predictors, an antimicrobial peptide gene signature together with the abundance of specific bacterial species (Ruminococcaceae and Sutterella) were associated with corticosteroid-free remission at week 52 and showed to be a promising tool to guide therapeutic decisions (Hyams et al., 2019).

However, precision medicine in IBD is still at its infancy. Most of the above-discussed studies were performed using small cohorts and at experimental level. None of these biomarkers has been validated and it is now ready to enter into clinical practice. Great economic resources are needed to make this step. The optimum would be to include the research of predictive biomarkers in clinical trial designs. Usually, in clinical trials, the target population is selected taking into account only clinical and demographic characteristics, with results that almost never overcome 50% of response. The capacity to include the tools provided by precision medicine for a more accurate patients' selection would greatly improve both clinical and endoscopic response to therapy.

Another important aspect to be considered is the absolute need of independent validation cohorts due to the risk of bias in big data analysis. For instance, gene expression patterns of $CD8^+$ T cells were initially reported to correlate with clinical outcomes of adult IBD patients (Lee et al., 2011). However, more recently, Gasparetto and colleagues were unable to validate the findings of an association between $CD8^+$ T-cell gene transcription and disease outcome in IBD (Gasparetto et al., 2021).

An integrative personal profiling including all the tools for precision medicine (**Figure 1**), such as pharmacogenomics, gene

expression profiling, proteomics (serum/tissues), metabolomics, immunoprofiling, microbiota analysis and imaging, can improve disease risk assessment, accuracy of diagnosis, disease monitoring and targeted treatments (Li-Pook-Than and Snyder, 2013). This is true for all the complex diseases, including IBD. Thus, we can imagine that, in the next future, a patient with a new diagnosis of IBD will undergo not only clinical, endoscopic and radiologic evaluation, but also transcriptomics, immunoprofiling and microbiota analysis. Altogether this information will be used to build-up a model for predicting individual risk and likelihood of response to specific therapies, with the potential to enable delivery of truly individualised IBD care.

AUTHOR CONTRIBUTIONS

IM literature search, wrote the paper; GM wrote the paper, critical revision of the manuscript.

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REVIEW
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Type I Interferons in Systemic Autoimmune Diseases: Distinguishing Between Afferent and Efferent Functions for Precision Medicine and Individualized Treatment

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A sustained increase in type I interferon (IFN-I) may accompany clinical manifestations and disease activity in systemic autoimmune diseases (SADs). Despite the very frequent presence of IFN-I in SADs, clinical manifestations are extremely varied between and within SADs. The present short review will address the following key questions associated with high IFN-I in SADs in the perspective of precision medicine. 1) What are the mechanisms leading to high IFN-I? 2) What are the predisposing conditions favoring high IFN-I production? 3) What is the role of IFN-I in the development of distinct clinical manifestations within SADs? 4) Would therapeutic strategies targeting IFN-I be helpful in controlling or even preventing SADs? In answering these questions, we will underlie areas of incertitude and the intertwined role of autoantibodies, immune complexes, and neutrophils.

Keywords: interferon, systemic lupus erythematosus (SLE), genetic polymorphism, interferon-stimulated genes (ISGs), polymorphonuclear neutrophils (PMN), keratinocytes, autoantibody (autoAb), systemic autoimmune diseases (SADs)

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INTRODUCTION

The interferon (IFN) response indicates a chain of molecular events in cells and tissues which comprises identification of genetic material by pattern recognition receptors (PRRs), signal transduction and initiation of IFN production, the response to IFN, and expression of IFN-stimulated genes which then exert their function and establish regulatory feed-forward loops (**Figure 1**). IFNs have been originally described in 1957 as substances interfering with viral replication (Isaacs and Lindenmann, 1957). Since then, a large body of data implicates IFN in responses to viral infections by direct activities on infected cells and by profoundly influencing the behavior of cells of innate and adaptive immune response (Biron, 2001). Beyond antiviral activities, IFNs are involved in several biological processes playing a role in infectious diseases, cancer,

Abbreviations: IFN, interferon; IFNAR, interferon-alpha/beta receptor; IFNGR, interferon-gamma receptor; IFNLR, interferon lambda receptor; IRF, interferon regulatory factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; Tyk, tyrosine kinase. Modified from (Hall and Rosen, 2010).

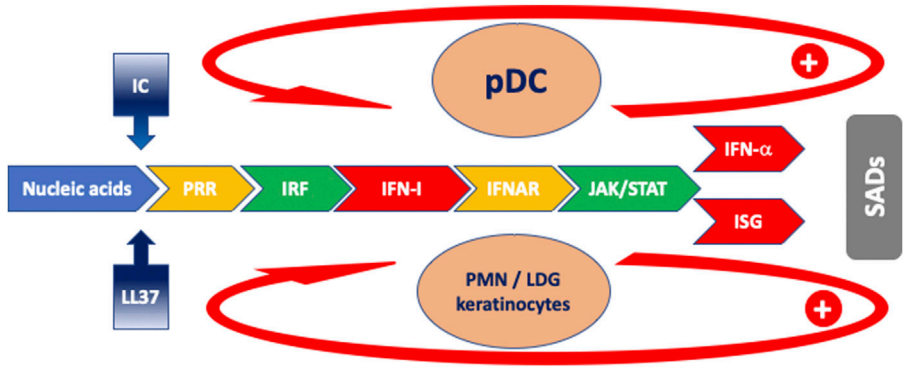

FIGURE 1 | Schematic model of the cascade of events characterizing the IFN response in SADs. Blue: antigen drive; gradient blue: facilitator mechanisms for antigen uptake; yellow: receptors; green: intracellular signaling; red: IFN and ISG. The arrows indicate feed-forward regulatory mechanisms. Orange ovals: main cells implicated in IFN-I production. Abbreviations: IC: immune complexes; IFNAR: interferon-alpha/beta receptor; ISG: interferon-stimulated gene; ISRE: IRF: interferon regulatory factor; JAK: Janus kinase; LDG: low-density granulocyte; LL-37: cathelicidin-37; pDC: plasmacytoid dendritic cell; PMN: polymorphonuclear neutrophil; PRR: pattern recognition receptor; SADs: systemic autoimmune diseases; STAT: signal transducer and activator of transcription.

TABLE 1 | Receptors and main signaling molecules used by IFNs.

	IFN-I	IFN-II	IFN-III
Receptor subunits	IFNAR1	IFNGR1	IFNLR1
	IFNAR2	IFNGR2	IL-10R2
Receptor expression	All nucleated cells	All nucleated cells	Epithelial cell pDCs
Signaling molecules	JAK1 and TYK2	JAK1 and JAK2	JAK1 and TYK2
Transcription factors	STAT1/STAT2/IRF9	STAT1/STAT1	STAT1/STAT2/IRF9
	STAT1/STAT1		STAT1/STAT1

inflammation, and autoimmunity. IFNs comprise a quite large family of proteins currently subdivided into type I IFN (IFN-I) including alpha (encoded by 13 distinct genes), -beta, -delta, -epsilon, -kappa and -omega families produced by almost all nucleated cell types and type III IFN (IFN-III) or IFN-lambda particularly produced by cells of hematopoietic origin and by epithelia at barrier surfaces. In contrast, the production of type II IFN (IFN-II) or immune interferon or interferon gamma is largely restricted to cells of lymphoid origin, particularly NK and T cells. All IFN-I signal through an invariant two-chain receptor expressed on most cell types. Similarly, IFN-II uses a dedicated two-chain receptor also expressed on most cell types. At variance, the IFN-III two-chain receptor is preferentially expressed on cells of epithelial origin and on plasmacytoid dendritic cells (pDCs). The three sets of IFN receptors converge toward the JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathways, which may account to some extent for the partially overlapping sets of genes activated in response to distinct IFNs (Hertzog et al., 2011; Weerd and Nguyen, 2012) (**Table 1**; **Figure 2**). While IFN-I and IFN-III ISG repertoires generally overlap, IFN-III signaling leads to a more sustained expression of ISGs, and in contrast to IFN-I, IFN-III does not induce the transcription of pro-inflammatory cytokines (Galani et al., 2017; Lazear et al., 2019).

Evidence linking IFN to autoimmunity was published first in 1969 when poly I:C injection, which in a sense mimics viral infection, was shown to enhance disease manifestations in the (NZB/NZW) F1 murine lupus model (Steinberg et al., 1969). Thereafter, increased levels of IFN biological activity were documented in the serum of individuals suffering from various systemic autoimmune diseases (SADs) including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and Sjogren's syndrome (SS) (Skurkovich et al., 1977; Hooks et al., 1979; Ytterberg and Schnitzer, 1982). In the following 40 years, it has been solidly established that systemic autoimmunity beyond SLE, RA, SSc, and SS to include myositis, mixed connective tissue disease (MCTD), and undifferentiated connective tissue disease (UCTD) is associated with conspicuous IFN biological activities (Higgs et al., 2011; Ekholm et al., 2016; Barturen et al., 2020). Further interest in IFN and SADs has spurred form the recent identification of monogenic disorders named interferonopathies characterized by high IFN production and clinical manifestations partly resembling those of SADs (Crow and Manel, 2015).

While IFNs are associated with SADs, the presence of high levels of IFNs is detectable in some but not all individuals suffering of SADs with frequencies of individuals with high IFN varying across the various SADs (Barturen et al., 2020). In the perspective of precision medicine, the identification of

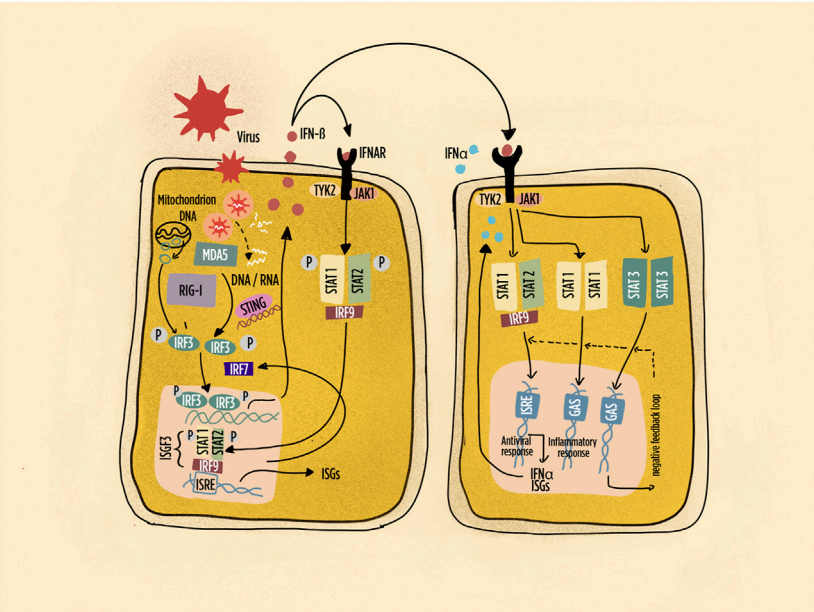

FIGURE 2 | Schematic representation of pathways leading to interferon (IFN) production and IFN responses in many cell types. Highlighted the intracellular sensors of viral and endogenous DNA/RNA; the main interferon regulatory factors; the primary response characterized by IFN-beta production with autocrine and paracrine responses. Abbreviations: IFNAR: interferon-alpha/beta receptor; ISGF: interferon-stimulated gene factor; ISRE: interferon-stimulated response element; IRF: interferon regulatory factor; JAK: Janus kinase; MDA5: melanoma differentiation–associated protein 5; RIG-1: retinoic acid inducible gene-1; STAT: signal transducer and activator of transcription; STING: stimulator of interferon genes; Tyk: tyrosine kinase. Dashed line: negative feedback response.

factors associated with high IFN may provide stratification of patients in order to offer them the most appropriate therapy. Excellent exhaustive reviews on the role of IFNs in SADs have been published in the last decade (Hall and Rosen, 2010; Ivashkiv and Donlin, 2014; Muskardin and Niewold, 2018; Crow et al., 2019; Rönnblom and Leonard, 2019). The present review will address succinctly several aspects linked with the role of IFNs in SADs taking SLE as prototypic for this class of diseases (Crow and Ronnblom, 2019). A particular attention will be devoted to mechanisms initiating IFNs production—which we name afferent function—and the role of IFNs in tissue pathology—to which we refer as efferent function. We will highlight the role of autoantibodies (autoAbs), immune complexes (ICs), and polymorphonuclear neutrophils (PMNs) in driving IFN production. While most of our attention will be dedicated to IFN-I, particularly to IFN-alpha, we will also review some aspects of IFN-II in SADs. Besides IFNs, many other factors play major roles in SADs with varying clinical and pathogenic associations (Kunz and Ibrahim, 2009; Simon et al., 2021). The complex mosaic of intervening cytokines is depicted in **Supplementary Figure S1**. Their description goes beyond the scope of the present review.

WHAT ARE THE MECHANISMS LEADING TO HIGH IFN-I IN SADS?

IFN-I Producing Cells

While almost all nucleated cells produce IFN-I including circulating leukocytes, pDCs expressing at their surface the inhibitory type II lectin receptor BDCA2 (blood dendritic cell antigen 2) are particularly potent and well-recognized producers of IFN-alpha (Rönnblom and Alm, 2001) (**Figure 3**). Of note, pDCs have been described infiltrating target organs in practically all SADs, thus reinforcing both the importance of IFN-alpha and of pDCs in immunopathology. Consistently with a central role of pDC in SLE, a recent phase 2 therapeutic trial assessing a monoclonal Ab ligating BDCA2 that inhibits the production of IFN-I and other inflammatory mediators has shown efficacy in reducing skin lesions and IFN signature in the blood (Furie et al., 2019a). PMNs have also been implicated in the production of IFN (Decker, 2011), particularly a subset named low density granulocyte (LDG) (Denny et al., 2010). PMN and LDG may participate to the IFN signature determined in peripheral blood and in tissue target of pathology (Kegerreis et al., 2019). This respect is relevant to stress that several cell types may contribute differentially to the production and IFN gene signature detected in blood. Thus, their relative numbers may affect the type and amount of detectable ISG. Keratinocytes and other epithelial cells are poised to respond and to produce IFNs. Characteristically, they produce IFN-III, but in addition, keratinocytes are high producers of IFN-kappa. Expression of interferon-kappa is significantly enhanced in keratinocytes upon viral infection, upon exposure to double-stranded RNA, or upon treatment with either interferon-gamma or interferon-beta (LaFleur et al., 2001). Most importantly, a very recent article reported primary production of IFN-kappa by keratinocytes in preclinical autoimmunity and SLE, simultaneously providing evidence for a functional impairment of pDC with defective production of IFN-

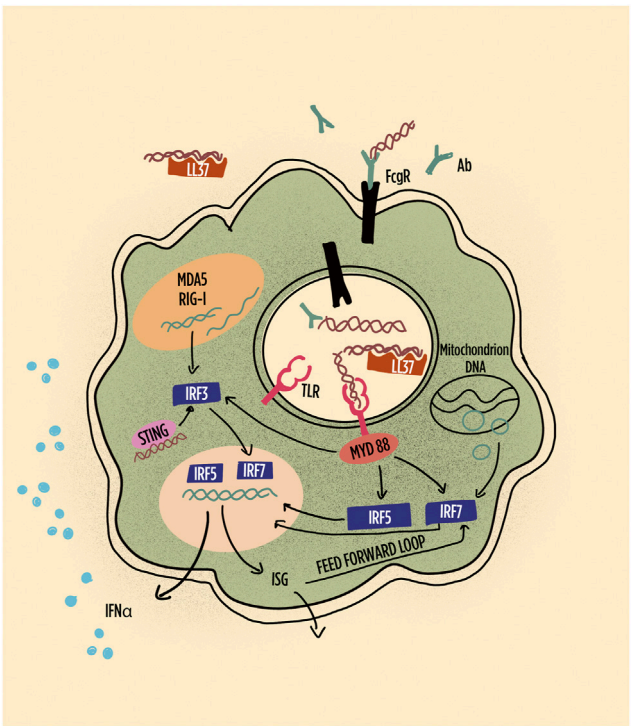

FIGURE 3 | Schematic representation of pathways leading to interferon (IFN) production and IFN responses in plasmacytoid dendritic cells (pDCs). Highlighted are the role of immune complexes and LL-37 in shuttling DNA/RNA into endosomes; the subsequent the role of TLR in inducing interferon regulatory factors and their role in gene transcription of IFN-alpha and other interferon-induced gene products, including feed-forward loops. RNA, particularly long double-stranded RNA, is preferentially sensed by MDA5 and RIG1. Abbreviations: Ab: antibody; FcgR: Fc gamma receptor; IRF: interferon regulatory factor; LL-37: cathelicidin-37; MDA5: melanoma differentiation–associated protein 5; MyD88: myeloid differentiation primary response 88; RIG-1: retinoic acid inducible gene-1; STING: stimulator of interferon genes; TLR: toll-like receptor.

α (Psarras et al., 2020). Consistently with these findings, when explored at single cell level by RNAsec, peripheral blood pDCs in SLE were found unable to produce IFN-α (Nehar-Belaid et al., 2020). These controversial findings highlight current difficulties in identifying the cells producing IFN-I in SADs.

Methods Used to Detect IFN-I

It has to be taken in mind that in most instances is not IFN *per se* that has been detected but rather the expression of genes that are induced by IFN and referred to as interferon-stimulated genes (ISG) or IFN gene signature. This approach reflects the span of biological processes initiated and maintained by IFNs with hundreds of genes activated in cascade. Potentially, 10% of our genome may respond to IFNs (Schoggins, 2019). The IFN signature overcomes the technical difficulty to detect low levels of the various IFN class members by solid phase assays that have low sensitivity and, the detection of IFN biological activity, which while possessing higher sensitivity, requires cumbersome procedures. Nonetheless, the drawback of using ISG as readout for IFN production is linked to the partial overlap in the genes induced by the three classes of IFN, which may confound and complicate the interpretation of the data generated (Hall et al., 2012). Furthermore, under certain circumstances, sustained expression of a subset of ISGs can take place over prolonged time periods, even in the absence of ongoing cytokine-mediated signaling (Cheon et al., 2013). A more recent methodology named SIMOA (single molecule array) based on the paramagnetic detection of single molecules complexed on beads has been used to detect IFN-alpha with a sensitivity in the femtogram per ml range (Wilson et al., 2016).

Mechanisms at Play in the Induction of IFN-I

Given the presence of IFNs in SADs, then the question arises about the mechanisms leading to IFN production in these pathological conditions. Type I and III IFN are physiologically produced when the presence of genetic material (DNA and RNA) of pathogen origin is sensed by specific receptors in the cytosol or in endosomes. However, also "self" DNA and RNA may activate such receptors when delivered in the appropriate manner (Barrat et al., 2005; Barrat et al., 2016; de Jong et al., 2016). Defective clearance of cells undergoing apoptosis or necrosis may provide the antigenic material composed of nucleic acids and nucleoproteins (Casciola-Rosen et al., 1994; Mahajan et al., 2016). Along the same line of evidence, polymorphisms of gene coding for enzymes deputed to DNA and RNA degradation are associated with an increased risk of SLE (Crow and Ronnblom, 2019). Some examples are polymorphisms of deoxyribonuclease 1 like 3 (*DNase1L3*), three prime repair exonuclease 1 (*TREX1*), SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (*SAMHD1*), and ribonuclease H2 subunit A (*RNASEH2A*) (Jiang et al., 2020). Of interest, the loss of function of these very same genes is associated with interferonopathies, stressing the role of nucleic acid in the induction of IFN-I (Rodero and Crow, 2016). Well-identified receptors for the genetic material are TLRs (toll-like receptors) anchored on cell membranes or more specifically on internal organelle membranes. Additionally, cytosolic receptors play important roles in recognizing nucleic acids. They include RIG-1 (retinoic acid inducible gene-1), MDA5 (melanoma differentiation–associated protein 5), NLR (nucleotide oligomerization-like domain receptor), and cGAS–STING (cyclic GMP-AMP synthase-stimulator of interferon genes) (Crow et al., 2019) (**Table 2**). IFN-II is produced in response to the immune activation of NK and T cells, with IFN-I, IFN-II, interleukin-12 (IL-12), and IL-18 playing a major role in the induction of IFN-II.

Immune complexes (ICs) containing DNA or RNA, eventually associated with nucleoproteins, are well documented and important inducers of IFN-I in SADs. Defective clearance of apoptotic material may provide the antigenic material targeted by autoAb forming these ICs (Casciola-Rosen et al., 1994). Defective digestion of extracellular genetic material may enhance this phenomenon (Sisirak et al., 2016). In these ICs, autoAbs are captured by FCgamma receptors at the cell surface and shuttled with their antigen in the endosomal compartment where they activate TLR7 (RNA) or TLR9 (DNA). This was initially

TABLE 2 | Main signaling pathways leading to IFN-I production.

Ligand	Receptor	Proximal transducing molecule	Transcription factors[a]
Extracellular DNA	TLR9[b]	MyD88	IRF5 and IRF7
Extracellular RNA	TLR7[b]	MyD88	IRF5 and IRF7
Extracellular dsRNA	TLR3[b]	TRIF	IRF3 and IRF7
Intracellular long dsRNA	MDA-5	MAVS	IRF3, IRF5, and IRF7
Intracellular short dsRNA	RIG-1	MAVS	IRF3, IRF5, and IRF7
ssRNA	NLR[c]		IRF3 and IRF5
Intracellular DNA	cGAS	STING	IRF3, IRF5, and IRF7

[a]*These transcription factors work in concert with additional molecules forming transcriptional complexes.*
[b]*Present in endosomes. The ligands need to be shuttled into endosomes to activate the receptors.*
[c]*NLRs comprise three families of proteins: NOD (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, and CIITA), NLRP (NLRP1-14, also referred to as NALP), and IPAF.; Abbreviations: cGAS: cGMP-AMP synthase; CIITA: class II, major histocompatibility complex, transactivator; IRF: interferon regulatory factor; MDA5: melanoma differentiation–associated protein 5; MAVS: mitochondrial antiviral signaling protein; MyD88: myeloma differentiation primary response 88; NLRs: nucleotide-binding oligomerization domain-like receptors; NLRP: nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (also abbreviated as NALP); NOD: nucleotide oligomerization domain; RIG-1: retinoic acid inducible gene-1; STING: stimulator of interferon genes; TLR: toll-like receptor; TRIF: TIR-domain–containing adapter-inducing IFN-beta. Primary data from (Zhao et al., 2015; Zarrin et al., 2020).*

demonstrated in SLE, SS, and SSc by Ronnblom and colleagues (Vallin et al., 1999; Bave et al., 2000; Bave et al., 2005; Kim et al., 2008). Furthermore, IFN-I in SLE serum was shown to capacitate the maturation of DCs from monocytes (Blanco et al., 2001), and DCs are fundamental to activate T cells and could favor the resurgence of autoreactive T cell clones which may at their turn provide appropriate help to autoreactive B cells. This may therefore link the enhanced production of IFN-I to adaptive immunity. However, the autoAbs recognizing self-DNA or RNA are themselves the product of adaptive immunity, which therefore should have preceded the formation of immune complexes. Thus, ICs potently enhance IFN-I production and participate to an amplification loop resulting in higher IFN-I production. A question that has not been addressed formally yet is whether autoAbs are needed for an IFN-I initial production. A corollary of this question is whether natural Abs, which are produced in the absence of antigenic stimulation and have low affinity and are polyreactive, may appropriately present self-nucleic acids to IFN-I producing cells (Madi et al., 2012).

A more recently described mechanism shown to favor the production of IFN-I by pDC involves the capacity of amphipathic peptides to form complexes with extracellular DNA or RNA allowing the shuttling of this material into endosomes (Lande et al., 2007; Ganguly et al., 2009; Lande et al., 2019). When properly directed into endosomes, the genetic material activates TLR7, TLR8, or TLR9. Notably, amphipathic peptides deliver DNA or RNA into endosomes as efficiently if not more compared to autoAb (Lande et al., 2019). Examples of these peptides are, among others, cathelicidin also known as LL-37 and chemokines such as CXCL4 also known as platelet factor 4 (PF4). LL-37 is produced mainly by keratinocytes and PMN, both important players in SLE pathogenesis. PF4, abundantly released by platelets, is produced by several cell types of hematopoietic origin. Most interesting, LL-37 decorates DNA when extruded by PMN forming extracellular traps (NETosis) (Kahlenberg and Kaplan, 2013). This active process characterizes SLE and many other SADs (Granger et al., 2019). Thus, PMNs undergoing NETosis provide both DNA and peptides that favor its entry in pDC resulting in enhanced IFN-I production (Lande et al., 2011). In the perspective of the present review, the question then arises whether in SLE and in other SADs the propensity of PMN to participate to disease pathogenesis is a primary or a secondary event (Wirestam et al., 2019). In other words, whether the activation and subsequent NETs formation by PMNs, which favor IFN-I production, is intrinsic to PMNs or whether they become activated because other pathogenic phenomena occur. For instance, it is well described that immune complexes have the capacity to activate PMNs which then undergo NETs formation (Lande et al., 2011). Furthermore, both LL-37 and CXCL4 were shown to be target of autoAbs that participate to both enhanced NETosis and enhanced stimulation of pDC to produce IFN-I. Indeed, genes associated with granulopoiesis were shown to be expressed in active SLE (Bennett et al., 2003) and characterize a subgroup of pediatric SLE (Banchereau et al., 2016), a robust finding confirmed when adult and pediatric SLE cohorts were pooled for the analysis (Toro-Dominguez et al., 2018). Both in SLE and in RA (Wright et al., 2017), low-density granulocytes (LDGs) have been identified which may represent a distinct cell population with specific functions. In SLE, but not in RA, the majority of LDGs, namely, the LDG expressing CD10, do not appear to be immature cells, but rather activated cells with enhanced expression of ISG, and enhanced function including enhanced NETosis, degranulation, chemotaxis, and release of oxidized mitochondrial DNA (Mistry et al., 2019). In SLE, CD10pos LDGs share with classical PMN enhanced expression of ISG. ISGs are not activated in immature CD10neg LDG (Mistry et al., 2019). Thus, a subset of LDG in SLE may represent an intrinsically pathogenic cell type, although this remains to be demonstrated. However, their number correlates with disease activity and organ damage (Mistry et al., 2019).

Several additional mechanisms may account for enhanced IFN-I production, in particular by pDCs. Briefly, they include activation by T cells, NK cells, B cells, and platelets each with a dedicated combination of signaling molecules (Rönnblom and Leonard, 2019). Recently, attention has been given to the capacity of mitochondrial DNA and long interspersed element 1, belonging to transposable DNA elements, to stimulate IFN-I

production by exploiting cytosolic DNA sensors (Lood et al., 2016; Mavragani et al., 2016).

WHAT ARE THE PREDISPOSING CONDITIONS FAVORING HIGH IFN-I PRODUCTION?

Gene Polymorphisms Modulating IFN-I Production and IFN Responses

Several gene polymorphisms associated with an increased risk of disease development are shared between SADs (Teruel and Alarcón-Riquelme, 2016). Of interest, many of them are localized in the IFN pathway (Jiang et al., 2020). These polymorphisms may modulate IFN production when signals are propagated.

IRF5

The signaling cascade initiated among others by TLR7 and TLR9 occupancy leads to IRF5 phosphorylation, homo- or heterodimerization, nuclear translocation, and binding to the promoters of type I IFN genes (**Figure 3**). IRF5 polymorphisms have been associated with SLE and several other SADs (Sigurdsson et al., 2005; Graham et al., 2007; Dieude et al., 2009; Miceli-Richard et al., 2009; Tang et al., 2014; Matta and Barnes, 2020). Consistently with the role of IRF5 and IFN-I in SLE, the lack of IRF5 prevents disease development in models of murine lupus (Richez et al., 2010). In SLE, enhanced levels of serum IFN-I have been associated with IRF5 polymorphisms (Niewold et al., 2008). Interestingly, however, high levels of IFN-I, assessed by a functional assay, were not uniformly distributed according to IRF5 polymorphisms. Rather different IRF5 haplotypes characterized by a different combination of functional genetic elements were associated or with anti–double-stranded DNA (dsDNA) or with anti-SSA/Ro52 autoAb (Niewold et al., 2012). Thus, the genetic risk was revealed by the presence of patient-restricted autoantibodies.

IFIH1

Similar findings were obtained when assessing the association between a polymorphism of IFIH1 (IFN induced with helicase C domain 1; also known as MDA5) and IFN-I serum levels in SLE (Robinson et al., 2011). IFIH1 is a cytoplasmic dsRNA sensor that activates IFN-α pathway signaling. In this case again, higher serum IFN-I levels were detected only in individuals with the appropriate allele and positive for DNA autoantibodies. We share with the authors the opinion that these data support a model in which autoAb and the formation of immune complexes then lead to enhanced IFN-I production. These data are consistent with the contention that adaptive immune responses precede enhanced production of IFN-I.

STAT4

STAT4 is activated downstream of a number of cytokines, including type I IFNs and contributes to T cell differentiation and IFN-γ production. Polymorphisms within STAT4 have been linked with an increased risk of RA, SLE, SSc, and SS (Remmers et al., 2007; Dieude et al., 2009; Nordmark et al., 2009; Gestermann et al., 2010). Furthermore, patients with SLE and the STAT4 risk haplotype have a more severe disease phenotype (Taylor et al., 2008). Of interest, a STAT4 variant (T allele; rs7574865) was reported to render SLE peripheral blood mononuclear cells more responsive to IFN-I as assessed by their expression of ISG (Kariuki et al., 2009). Further, the same variant was associated with enhanced production of IFN-gamma by CD4 and CD8 T cells in response to both IFN-I and IL-12 (Hagberg et al., 2018). These data provide evidence that polymorphisms downstream IFN-I signaling, therefore belonging to their efferent function, have functional consequences. Interestingly, they appear to participate to enhance IFN-II production.

Downstream Effects and Regulation of IFN-I Signaling

The first consequence of IFN-I production is an enhanced production of IFN-I itself (**Figure 1**). This feed-forward loop, elegantly discussed by John C. Hall and Antony Rosen (Hall and Rosen, 2010), results in rapid amplification of IFN-I responses. Mechanistically, many of the receptors, which include cytoplasmic and endosome sensors of nucleic acids, signal transduction molecules and transcription factors that drive IFN production are themselves regulated by IFNs. These feed-forward loops favor the expression of hundreds of genes and create the potential for amplifying immunopathology in SADs by affecting the function of target cells in the tissues and by modulating the activity of antigen presenting cells and effector cells of the immune system (Biron, 2001). Upon the initial induction of IFN-I production, remarkable are the explosive production of IFN-I by pDC and antigen presentation by conventional DC, including the expression of co-stimulatory molecules and the activation of potentially autoreactive T and B cells. Further, several autoantigen targets of autoAb are highly responsive to IFN, potentially augmenting antigen drive in SADs. Among others, the expression of the autoantigen SSA/Ro 52 kD, which has direct antiviral properties, increases in cells under the influence of IFN-I enhancing the presentation of immunostimulatory Ro52 epitopes (Strandberg et al., 2008). Indeed, the autoantigen SSA/Ro 52 is targeted by autoAb in several SADs, with the titer of anti-SSA/Ro autoAb remaining stable during disease evolution.

IRF3 activation and IFN-β production are observed in most cells at the initiation phase of the IFN-I response (**Figure 2**). The autocrine and paracrine activity of IFN-β then induces the expression of IRF7, which positively regulates IFN-I production in adjacent cells. Of interest, pDCs constitutively express high levels of IRF7, thus explaining their rapidly and potent response to activation. In addition, pDCs express IRF5, which induces the transcription of IFN-α genes distinct from those induced by IRF7 (Barnes et al., 2004). Indeed, pDCs have been identified in target organs of virtually all SADs, and IRFs are critical regulators of the quality and quantity of IFN-I responses (Jensen and Niewold, 2015; Ye et al., 2020). The response to IFNs,

mediated by the canonical JAK-STAT signaling transduction pathway, is further modulated by the composition of the molecular complexes involved in gene transcription (**Table 1**). Thus, the interferon-stimulated gene factor 3 (ISGF3) complex (composed of STAT1, STAT2, and IRF9) activates classic antiviral genes. By contrast, STAT1 homodimers induce pro-inflammatory gene expression, and STAT3 homodimers suppress pro-inflammatory gene expression (Ivashkiv and Donlin, 2014), participating to downregulating regulatory loops. Additional complexity is provided by the contribution of signaling pathways involving MAPK (mitogen-activated protein kinase), NFkappaB (nuclear factor kappa-light-chain-enhancer of activated B cells), and PKB (protein kinase B) which may influence the composition of ISGF and the consequent activation of specific genes triggered by IFN-I. Host factors such as the concurrent presence of inflammatory cytokines or chemokines therefore participate to the modulation both positive and negative of IFN-I signaling (Ivashkiv and Donlin, 2014). It is worth to stress here that IFN-I and TNF tend to cross talk resulting in reciprocal inhibition (Banchereau et al., 2004). Indeed, therapies based on TNF blockade may result in enhanced expression of ISG in the peripheral blood (Mavragani et al., 2007), a mechanism potentially at play in TNF blockade–induced SLE. It is therefore remarkable that the tyrosine-protein phosphatase non-receptor type 22 (PTPN22) C1858T polymorphism is associated with skewing of cytokine profiles toward high IFN-I activity and low TNF levels in patients with SLE (Kariuki et al., 2008). However, in synovial RA macrophages, TNF drives ISG expression, but at the same time, it limits type I IFN-mediated signaling and modulates the pattern of ISG expression (Gordon et al., 2012). Further, TNF may participate to IFN-beta induction by IRF1 signaling (Yarilina et al., 2008).

Overall, the different IFN types and subtypes participating to responses and the cell intrinsic and distinct temporal distribution of molecular complexes involved in IFN intracellular signaling concur in modulating their effects on the quality and quantity of gene transcribed, which may account for the IFN heterogeneous biological and pathological effects.

WHAT IS THE ROLE OF IFN-I IN THE DEVELOPMENT OF DISTINCT CLINICAL MANIFESTATIONS WITHIN SADS?

Systemic Autoimmune Diseases Heterogeneity and IFN-I

Clinical manifestations and biological abnormalities allow to distinguish between SADs and classification criteria perform well enough to group patients with diverse and different disease manifestations. To a large extent, these differences lead to different therapeutic strategies applied to SADs. This notwithstanding, a common set of 36 type I IFN inducible transcripts was identified among the most overexpressed in the whole blood of 262 patients with SLE, RA, SSc, and myositis in contrast to 26 healthy controls (Higgs et al., 2011). Along the same line of evidence, when unsupervised clustering of integrated whole blood transcriptome and methylome was performed with data of 263 healthy controls and 918 patients with seven SADs (SLE, RA, SS, SSc, MCTD, antiphospholipid syndrome, and UCTD), among the four identified clusters, the "interferon" cluster was grouping individuals with all seven distinct SADs (Barturen et al., 2020). Thus, an IFN signature is common to all SADs, which associated with the genetic polymorphisms of IFN-related genes shared between SADs, may account for part of the "heritability" of systemic autoimmunity (Niewold et al., 2007; Kariuki et al., 2010). Beyond this commonality, subtler analyses may provide important information accommodating heterogeneity in clinical manifestations between and within SADs. In this respect, the subdivision of ISG modules based on complex correlations and factor analysis within expressed genes resulted in two simplified IFN scores that allowed categorization of SLE vs. RA (El-Sherbiny et al., 2018).

Systemic lupus erythematosus. Therapeutic use of IFN-α, for instance, in the setting of chronic hepatitis C infection, may lead to clinically overt SLE, which regresses after therapy suspension (Niewold and Swedler, 2005). Consistently with a pathogenic role, a rise in circulating IFN-I precedes disease manifestations in SLE and accompanies disease severity (Bengtsson et al., 2000; Baechler et al., 2003; Kirou et al., 2005; Munroe et al., 2016). However, an IFN signature is found in only 50–80% of SLE patients (Baechler et al., 2003; Bennett et al., 2003; Crow and Wohlgemuth, 2003) and the IFN-induced gene signature assessed in longitudinal studies may not correlate with disease activity (Landolt-Marticorena et al., 2009; Petri et al., 2009). Indeed, in addition to IFN modules, other gene modules have been variably reported to associate with SLE clinical features (Banchereau et al., 2016; Rai et al., 2016; Lu et al., 2019; Barturen et al., 2020; Guthridge et al., 2020). These data indicate that IFN does not account for all pathological and clinical aspects of SLE, which may be further explained by heterogeneity in the IFN-I pathway activation and genetic makeup (Kariuki et al., 2015). In a pediatric SLE population undergoing frequent relapses, the modules of IFN-I-related genes were among the most prevalent with those related to the myeloid lineage (Banchereau et al., 2016). In accordance with others, we found that in multivariate analysis, only mucocutaneous and articular SLE clinical manifestations were specifically associated with high IFN-I gene signature detected in peripheral blood (Chasset et al., 2020). Of interest, the clinically most active patients combined higher expression of IFN-I and PMN genes in peripheral blood (Chasset et al., 2020). To be noted, however, that the risk of relapse appears to increase in SLE patients with high vs. low IFN-alpha levels, when assessed by SIMOA (Mathian et al., 2019). In addition, when assessing gene expression, a relationship between inactive vs. moderately active or very active disease was found with diverse modules of expressed genes, with a contribution by IFN-beta and IFN-gamma in addition to IFN-alpha (Jourde-Chiche et al., 2016). Similarly, high levels of circulating interferons type I, type II, and type III were found to be associated with distinct clinical features of active SLE (Oke et al., 2019). IFN-kappa expressed in keratinocytes and *IFNk* gene polymorphisms in SLE appear to be involved in cutaneous manifestations accelerating

responsiveness of epithelia to IFN-α and increasing keratinocyte sensitivity to UV irradiation. (Harley et al., 2010; Sarkar et al., 2018). IFN-III also appears to have a role in SLE skin lesions (Zahn et al., 2011). "Natural autoantibodies" directed against IFN-alpha have been reported in SLE positively correlating with disease activity (Gupta et al., 2016). However, a subset of these were blocking autoAb and were associated with the absence of IFN gene signature and reduced SLE disease activity (Gupta et al., 2016). Of great interest, in a longitudinal study addressing the presence of IFN in the sera of individuals which would develop SLE, the presence of IFN-II and of chemokines induced by IFN-II temporally preceded the detection of IFN-I itself associated to the increased presence of autoAb directed against nucleoproteins or DNA. The clinical manifestations then followed (Munroe et al., 2016). Within the limits of the relatively low number of individual tested and the sensitivity of the assays used to detect IFN-I and IFN-II, this is an important piece of evidence indicating that an adaptive immune response in SLE precedes and accompanies the initial detection of IFN-I (Lu et al., 2016). Along the same line of evidence, clinical responders, as opposed to nonresponders in a phase 2 trial assessing the efficacy and safety of ustekinumab (anti-IL-12/IL-23) in SLE had treatment-dependent reduced serum levels of IFN-gamma and not of other cytokines (van Vollenhoven et al., 2018; Cesaroni et al., 2020; van Vollenhoven et al., 2020).

Sjögren syndrome. SS classical clinical manifestations include dry eye and dry mouth due to exocrine gland inflammation. However, systemic manifestations are frequent with involvement, among others, of the peripheral nervous system, skin, and kidneys distinctly different from those observed in SLE. However, SS very much resemble SLE in terms of IFN-I signature detected in peripheral blood and shared genetic risk factors. Thus, in SS, a peripheral blood IFN-I gene signature strongly correlates with the presence of anti-SSA/Ro 52 autoAb (Emamian et al., 2009). Of interest, studies of minor salivary glands revealed both enhanced IFN-I and IFN-II gene signatures with IFN-II being predominant and associated with lymphomagenesis risk (Nezos et al., 2015). In addition, in salivary glands, epithelial cells were contributing to IFN-beta and infiltrating pDC to IFN-alpha production (Mavragani et al., 2016).

Myositis. Primary inflammatory myositis comprise a large array of distinct clinical syndromes in which muscle inflammation is often accompanied by skin, joint, lung, vascular, and other abnormalities. Perhaps, myositis as a whole is the SAD with the best correlation between IFN-I levels and disease activity. In myositis, IFN-I levels are increased in the circulation and most interestingly in muscle tissue with an association with disease activity (Niewold et al., 2009; Greenberg et al., 2012). Along the same line of evidence, pharmacological inhibition of JAK signaling in a clinical trial improved IFN-I–induced muscle fiber damage (Ladislau et al., 2018). Studies of muscle biopsy showed that immature muscle precursor cells overexpressing HLA class I are a source of IFN-beta, which may play a direct role in the induction of IFN-I signature in muscle fibers (Tournadre et al., 2012). Serum IFN-beta rather than IFN-alpha levels appear to correlate with cutaneous manifestations and their severity in dermatomyositis (Huard et al., 2017). Further, IFN-I enhances the expression of some autoantigens including MDA5 which defines a very specific clinical subtype of dermatomyositis, thus reinforcing a pathogenic role of IFN-I in myositis subsets (Sato et al., 2009; Fiorentino et al., 2011).

Systemic sclerosis. SSc is characterized by fibrosis of the skin and internal organs including the lung, the gastrointestinal tract, and heart, accompanied by prominent vasculopathy. SSc shares with the other SADs, and with SLE in particular, both high IFN-I gene signature in peripheral blood (Assassi et al., 2010) and gene polymorphisms of the IFN pathway linked to increased risk of disease. Of pathogenic interest, the IFN-I gene signature may precede the development of lung fibrosis (Brkic et al., 2016). Peculiar to SSc, the very high serum levels of CXCL4, which are associated to lung fibrosis and pulmonary arterial hypertension (van Bon et al., 2014). CXCL4 acts as a chaperone shuttling extracellular DNA into endosomes in pDC enhancing in TLR8- and TLR9-dependent manner the production of IFN-I (Ah Kioon et al., 2018; Lande et al., 2019).

Rheumatoid arthritis. RA is mainly characterized by erosive symmetrical arthritis, but systemic manifestations may involve the lung, the skin, and other organs. While the IFN-I signature is less conspicuous in RA than other SADs including SLE (Higgs et al., 2011; Barturen et al., 2020), an IFN-I signature precedes overt clinical manifestations and its presence increases the risk of developing the disease (Lübbers et al., 2013). The presence of pDCs and the expression of ISGs, IFN-alpha, and IFN-beta have been documented in the synovium of patients with RA (Lande et al., 2004; van Holten et al., 2005). Of interest, monocytes, chondrocytes, and fibroblast-like synoviocytes were shown to respond to IFN-beta by enhanced production of the anti-inflammatory of IL-1 receptor antagonist (Coclet-Ninin et al., 1997; Palmer et al., 2004), which may counteract the potentiation by IFN-alpha of the TLR4-mediated production of IL-1-beta in RA synovial cells (Roelofs et al., 2009). In relationship with these observations, two independent studies consistently reported that the response to tumor necrosis factor (TNF) inhibitors in RA was predicted by the ratio of pretreatment IFN-beta to IFN-α activity, with lower ratios (higher IFN-beta) associated with responses to TNF inhibition (Mavragani et al., 2010; Wampler Muskardin et al., 2016). To extend these findings, the ratio between IFN-alpha and IFN-beta appears to vary in different SADs, suggesting a complex participation of these two IFN subtypes to autoimmunity (de Jong et al., 2016).

WOULD THERAPEUTIC STRATEGIES TARGETING IFN-I BE HELPFUL IN CONTROLLING OR EVEN PREVENTING SADS?

Better understanding for the role of IFN-I in SADs has led to a wide array of therapeutic strategies aiming at blocking, neutralizing, and inhibiting IFN-I or inhibiting intracellular signaling initiated by IFN receptor engagement or targeting high IFN-I–producing cells (Chasset and Arnaud, 2018; Jiang

TABLE 3 | Clinical trials of molecules targeting IFN-I, cells producing IFN-I, or IFN-I–related signaling pathways in clinical development ≥ phase two trials in lupus erythematosus.

Type of inhibitor	Name	Current developmental phase	Primary outcome achieved	Main outcome	Refs
Anti–IFN-α mAb	Rontalizumab	Phase 2	No	BILAG at w24	Kalunian et al. (2016)
	Sifalimumab	Phase 2	Yes	SRI-4 at w52	Khamashta et al. (2016)
Therapeutic vaccine IFN-α	**Interferon-α-kinoid**	**Phase 2b, ongoing phase 3**	**No**	**Modified BICLA at w36**	Houssiau et al. (2020)
Anti-IFNAR1 mAb	**Anifrolumab**	**Phase 3**	**Yes/ No**	**SRI-4/BICLA at w52**	Furie et al. (2019b); Morand et al., (2020)
Anti-pDC	**BIIB059**	**Phase 2, ongoing phase 3**	**Yes**	**% Change in CLASI-A at w16**	Furie et al. (2019a)
JAK1/JAK2 inhibitors	**Baricitinib**	**Phase 2, ongoing phase 3**	**Yes**	**Arthritis/rash SLEDAI-2K at w24**	Wallace et al. (2018)
JAK1/JAK3 inhibitors	**Tofacitinib**	**Ongoing phase 2 in CLE**	**NA**		
JAK1/JAK2/JAK3 inhibitor	Tanzisertib	Phase 2	No	NA	
JAK1 selective inhibitor	Solcitinib	Phase 2	No	NA	
	Filgotinib	**Phase 2 in CLE**	**No**	**% Change in CLASI-A at w12**	
Topical JAK/SYK inhibitor	R333	Phase 2	No	≥50% decrease CLASI-A at w4	Presto et al. (2018)
Tyk-2 inhibitor	**BMS-986165**	**Ongoing phase 2**	**NA**		
Syk inhibitors	**Lanraplenig**	**Phase 2**	**NA**		Blomgren et al. (2020)
	Fostamatinib	Phase 2	NA		
BTK inhibitors	**Evobrutinib**	**Phase 2**	**NA**		Haselmayer et al. (2019)
	Elsubrutinib ABBV-105	**Phase 2**	**NA**		Goess et al. (2019)
	Fenebrutinib	**Phase 2**	**No**	**NA**	Lorenzo-Vizcaya et al. (2020)
TLRs 7, 9 inhibitors	DV1179	Phase 2a	No	NA	

In bold, molecules currently in continuous clinical development. BTK: Bruton tyrosine kinase; IFN: interferon; IFNAR1: interferon-alpha receptor 1; JAK: Janus kinase; pDCs: plasmacytoid dendritic cells; SYK: spleen tyrosine kinase; TLR: toll-like receptor; Tyk 2: tyrosine kinase-2.

et al., 2020). Since the proximal intracellular signaling pathways are shared by IFNs with several other cytokines, the expected inhibitory effects of intracellular signaling inhibition are broader than those resulting from direct IFN or IFN receptor inhibition (Kubo et al., 2018). Indeed, it has been estimated that more than 40 types of cytokines transmit signals through the JAK/STAT pathway (Kubo et al., 2019). On these bases, the inferences on the role of IFNs in SADs that can be deduced from therapeutic approaches will be dependent on the inhibition strategy and may vary substantially. Here, we provide a non-exhaustive overview of therapeutic approaches currently being tested in clinical trials, highlighting the differences between the various treatment strategies. The molecules under current testing in clinical trials having reached at least phase 2 levels are reported in **Table 3**.

Anti–IFN-Alpha Monoclonal Antibodies

The recombinant technology offers the possibility to raise high-affinity, neutralizing monoclonal antibodies (mAbs) against IFN-alpha. The specific difficulty here is linked to the fact that there are 13 different subtypes of IFN-alpha, with 75–99% amino acid sequence identity and different affinities for their receptor (Gibbert et al., 2013). While the mAbs which underwent clinical development were claimed to bind to and neutralize the majority of IFN-alpha subtypes, most likely they did not have the possibility to neutralize all the IFN-alpha biological activity. Further, this approach did not neutralize other type I IFNs which could have relevant pathological activities in SADs. However, interesting but somehow discouraging results were obtained in clinical trials in which anti–IFN-alpha mAbs were tested in myositis, SSc, and SLE. A phase 1b randomized, placebo controlled, clinical trial was conducted to evaluate sifalimumab (MEDI-545) in dermatomyositis (n = 27) and polymyositis (n = 21). Sifalimumab suppressed the type I IFN gene signature by 66% in the blood and 47% in the muscle at day 98. The authors reported a positive correlative trend between target neutralization and clinical improvement (Higgs et al., 2014), suggesting that direct type I IFN-I inhibition may be efficacious in myositis. To the best of our knowledge, however, no other clinical trials are currently conducted with this molecule in myositis. Rontalizumab and sifalimumab both reached phase 2 (Jiang et al., 2020) in clinical trials in SLE. Rontalizumab decreased the expression of ISG in phase 1 study with an acceptable safety profile (McBride et al., 2012). However, in the phase 2 study (*See* **Supplementary Table S1** for definitions of BILAG and other terms), the efficacy response rates assessed by the British Isles Lupus Assessment Group (BILAG)–based Composite Lupus Assessment (BICLA) (primary endpoint) and the Systemic Lupus Erythematosus Responder Index (SRI)-4 at week 24 (secondary endpoint) were similar between rontalizumab and placebo, and its development was terminated (Kalunian et al., 2016). Sifalimumab, a fully humanized IgG1 kappa anti–IFN-α mAb demonstrated in a phase 2b study higher SRI-4 response

index at week 52 than placebo. Of interest, sifalimumab efficacy was statistically significant in patients with high but not with low IFN-I gene expression signature, which hints to the advantage of selecting patients for this therapeutic approach (Khamashta et al., 2016).

Interferon-α-Kinoid

An alternative strategy explored the potential of neutralizing IFN-I by eliciting an endogenous immune response against IFN-I. This requires to break tolerance against self-IFN and induce autoimmunity. It was achieved with a therapeutic vaccine named interferon-α-kinoid (IFN-kinoid) composed of IFN-α-2b coupled to a carrier protein containing T helper cell epitopes that induces polyclonal anti-IFN-α neutralizing antibodies. In a phase 1/2 study, IFN-kinoid–induced anti–IFN-α antibodies in all immunized patients and significantly reduced the expression of the IFN gene signature compared to placebo (Ducreux et al., 2016). In a phase IIb study, 91% of immunized individuals having received five doses of vaccine developed detectable neutralizing antibodies. Overall, the IFN high gene expression signature decreased by 31%, but in 20/87 individuals with low titers of anti–IFN-alpha Ab (20/87), the IFN gene signature actually increased during the trial period. In the whole population, modified BICLA responses at W36 did not statistically differ between IFN-kinoid (41%) and placebo (34%) (Houssiau et al., 2020). However, attainment of lupus low disease activity state (LLDAS) at W36 discriminated the two groups in favor of IFN-kinoid (53 vs. 30%, $p = 0.0022$) with a significant glucocorticoid sparing effect. These analyses restricted to the subgroup of individuals having developed detectable anti-IFN-alpha antibody were all statistically significant. Interestingly, the immune response elicited by IFN-kinoid was not restricted only to IFN-α-2b but encompassed with variable efficacy also other members of the IFN-alpha subfamily in 50% of immunized individuals (Houssiau et al., 2020). No anti–IFN-beta Abs were found. Of further interest, IFN-kinoid revealed that IFN-α blockade had an inhibitory effect on the expression of B cell associated transcripts which highlight the intricate relationship between IFN-I and the adaptive immune response (Ducreux et al., 2016).

Anti-type I Interferon Receptor Antibodies

By targeting the common type I IFN alpha receptor 1 (IFNAR1) chain used by all IFN-I (13 IFN-alpha, IFN-beta, -delta, -epsilon, -kappa, and -omega), it is expected to obtain a broader IFN-I inhibition than anti–IFN-alpha mAbs and IFN-kinoid. Anifrolumab (MEDI546) is a fully human IgG1κ, effector null, monoclonal antibody directed against IFNAR1. *In vitro*, anifrolumab was shown to block IFN-I–dependent STAT1 phosphorylation and IFN-dependent signaling induced by IFNs and serum of patients with SLE. Anifrolumab suppressed IFN-I production by blocking the IFN autoamplification loop and inhibited pro-inflammatory cytokine induction and the upregulation of costimulatory molecules on stimulated pDCs. Blockade of IFNAR1 suppressed plasma cell differentiation in pDC/B cell co-cultures. (Riggs et al., 2018).

Systemic lupus erythematosus (SLE). In a phase 2b study (MUSE), the proportion of SLE patients who reached the SRI-4 primary outcome at week 24 were higher in those treated with anifrolumab (34.3% for 300 mg dose and 28.8% for 1,000 mg dose) than placebo (17.6%) ($p = 0.014$ and $p = 0.063$, respectively). The response was driven by the effect of anifrolumab in IFN high patients (Furie et al., 2017). Two phase 3 trials, TULIP 1 (Furie et al., 2019b) and TULIP 2 (Morand et al., 2020), testing the efficacy of anifrolumab added to the standard of care in active SLE were recently published. These trials had globally a similar design and most patients received intravenous anifrolumab (300 mg) or placebo every 4 weeks for 48 weeks. In TULIP 1, the proportion of patient reaching the SRI-4 primary outcome were similar between anifrolumab 300 mg (65 [36%] of 180) and placebo (74 [40%] of 184; difference −4·2 [95% CI: −14·2 to 5·8], $p = 0·41$). However, a BICLA response was achieved by 37% patients receiving anifrolumab vs. 27% receiving placebo (difference 10·1% [95% CI 0 6–19·7]). Conversely, in TULIP 2, BICLA response used as primary outcome was reached by 47.8% in the anifrolumab group and 31.5% in the placebo group (difference, 16.3% [95% CI: 6.3–26.3]; $p = 0.001$). In TULIP 2, the proportion of patients reaching the SRI-4 was also significantly higher in the anifrolumab group with a difference than placebo of 18.2% [95% CI: 8.1, 28.3] (Morand et al., 2020). In TULIP 1, the response at week 52 in patients within IFN gene signature high did not differ between the anifrolumab and placebo groups. In TULIP 2, 48.0% in the anifrolumab group and 30.7% in the placebo group, were responders in patients with a high IFN gene signature. The respective figures in patients with a low IFN gene signature were 46.7 and 35.5%, respectively. Of note, the frequency of IFN signature high patients in the aforementioned trials was >75%, conferring higher statistical power to the analysis of these patient groups. Across all clinical trials targeting IFN-I in SLE, significant efficacy was particularly evident on lupus mucocutaneous manifestations. Across all clinical trials, *herpes zoster* and respiratory tract infections were significantly higher or tended to be higher in patients receiving active compounds than patients receiving placebo.

Systemic sclerosis (SSc). A phase 1 open-label trial was conducted with anifrolumab in 34 adult SSc patients. In this study, anifrolumab rapidly induced a reduction of IFN gene signature in IFN-high individuals both in blood and skin (Goldberg et al., 2014). SSc patients with high IFN-I signature had significantly higher skin thickness than IFN-low patients, suggesting an association between high IFN-I signature and SSc severity. Moreover, anifrolumab administration was associated with significant downregulation of T cell-associated proteins and upregulation of type III collagen degradation marker, but no data on clinical findings were reported (Guo et al., 2015). To our knowledge, no other treatment targeting directly IFN-I is under development in SSc.

Targeting IFN-Beta

A small phase 2 randomized placebo-controlled trial evaluating the effect of PF-06823859 an IFN-beta 1 blocker is ongoing in

TABLE 4 | Current phase of development of Janus kinase inhibitors in systemic autoimmune diseases (based on https://clinicaltrials.gov/accessed on November 11, 2020).

Molecule name; [main target(s) of inhibition]	SLE	SS	DM/PM	SSc	RA
Baricitinib [JAK1, 2]	Phase 3 R	– –	Phase 2 NYR	–	Approved
Filgotinib [JAK1]	Phase 2 R	Phase 2 R	–	–	Approved
Peficitinib [JAK1, 2, 3]	–	–	–	–	Approved
Tofacitinib [JAK 1, 2, 3]	–	–	Phase 1 C	Phase 1/2 C	Approved
Upadacitinib [JAK1, (2)]	Phase 2 R	–	–	–	Approved
Ruxolitinib [JAK1, 2]	–	–	–	–	–

C: completed; DM: dermatomyositis; JAK: Janus kinase; NYR: not yet recruiting; PM: polymyositis, R: recruiting; RA: rheumatoid arthritis; SS: Sjogren syndrome; SSc: systemic sclerosis.

dermatomyositis (clinical trial NCT03181893). The rationale of this unique study may rely on findings, indicating that serum IFN-beta rather than IFN-alpha levels appear to correlate with cutaneous manifestations and their severity in dermatomyositis (Huard et al., 2017).

Targeting pDCs

The cells with the highest potential for IFN-alpha production are pDCs, and they play a central role in SAD pathogenesis. Therapeutic interventions aiming at decreasing pDC numbers or functions may result in decreased IFN-alpha production, in addition to decreased production of many inflammatory cytokines and decreased availability of co-stimulatory molecules. BIIB059 is a mAb that binds to BDCA2, an inhibitory receptor expressed on pDC surface, and induces its rapid internalization inhibiting the production of IFN-I. In a phase 1 trial including 12 SLEs, BIIB059 decreased the expression of IFN response genes in blood and improved the Cutaneous Lupus Erythematosus Disease Area and Severity Index Activity (CLASI-A) (Furie et al., 2019a). LILAC (NCT02847598) was a 2-part, phase 2 study investigating the efficacy and safety of BIIB059 was presented at the ACR Convergence 2020 meeting. BIIB059 (50, 150, and 450 mg) or placebo was subcutaneously administered once every 4 weeks in patients with cutaneous lupus erythematosus (CLE) with or without SLE, and all doses showed higher percent changes in CLASI-A than placebo (mean difference ranging from −24.29 to −33 (Werth et al., 2020b). A phase 3 study of BIIB059 is expected to start soon in cutaneous lupus erythematosus (CLE) with or without associated SLE.

Targeting Signaling Initiated by TLRs and IFN-I Receptor Occupancy

Molecules targeting signaling initiated by TLRs and IFN-I receptor occupancy which have reached at least phase 2 in clinical development are reported in **Table 4**.

JAK Inhibitors

Currently several JAK inhibitors (JAKis) based on small molecules are being developed in SADs including tofacitinib, baricitinib, upadacitinib, filgotinib, itacitinib, and peficitinib. Tofacitinib has been approved for RA, PsA, and ulcerative colitis, and other JAKis have been approved for RA. JAKis are also under development in phase 3 studies for atopic dermatitis, psoriasis, and alopecia areata (Nash et al., 2020). The current stages of development of main JAKi under development in SADs are presented in **Table 4**.

Systemic lupus erythematosus (SLE). In a SLE phase 2 randomized controlled trial, baricitinib treatment induced significant reduction in the RNA expression of a network of genes associated with the JAK/STAT pathway, cytokine signaling, and SLE pathogenesis. In addition, baricitinib consistently reduced serum levels of two key cytokines implicated in SLE pathogenesis, IL-12p40, and IL-6 (Dörner et al., 2020). Baricitinib at 4 mg/day but not at 2 mg/day showed higher resolution of SLEDAI arthritis and rash at week 24 than placebo: 70 (67%) of 104 patients receiving baricitinib 4 mg vs. 56 (53%) in the placebo group (odds ratio [OR] vs. placebo 1·8, 95% CI: 1.0–3.3; p = 0·0414). Of note, serious infections were reported in six (6%) patients with baricitinib 4 mg, two (2%) with baricitinib 2 mg, and one (1%) with placebo (Wallace et al., 2018). Three phase 3 trials with baricitinib (NCT03843125, NCT03616912, and NCT03616964) are ongoing in SLE patients. Other JAKis are in early development phase or failed to demonstrate efficacy in SLE. Indeed, tanzisertib (JAK1/JAK2/JAK3 inhibitors) and solcitinib (JAK1 selective inhibitor) development were stopped in phase 2 (Jiang et al., 2020). Tofacitinib (a pan JAKi) with high degree of selectivity against JAK1 and JAK3 more than TYK2 or JAK2 is in early phase 2 development in CLE (NCT03288324 and NCT03159936, recruiting). Filgotinib used in association with lanraplenib (Syk inhibitor) in a phase 2 randomized controlled trial failed to demonstrate superiority over placebo (unpublished data). Finally, upadacitinib (JAK1, +/−JAK2) inhibitor is currently in a phase 2 study (recruiting) with or without association with elsubrutinib (BTK inhibitor).

Sjögren syndrome (SS). In the systematic review of ongoing clinical trial in SS, Felten et al. identified that JAKi may be a potential treatment in SS. Indeed, filgotinib a selective JAK1 inhibitor (Mease et al., 2018) is being evaluated in an ongoing phase 2 trial. In the same trial, two other arms are evaluating GS-9876, a (Syk inhibitor) and tirabrutinib (BTKi).

Rheumatoid arthritis (RA). As introduced earlier, tofacitinib, baricitinib, upadacitinib, filgotinib, and peficitinib showed efficacy in phase 3 clinical trials in RA (Jiang et al., 2020). However, the main mechanism of action of JAKi in RA is probably not related to IFN-I inhibition. Syk inhibitors [lanraplenib (GS-9876)] and BKT inhibitors [spebrutinib (CC-292) and ABBV-105] are currently in phase 2 of development (Jiang et al., 2020).

Myositis. It has been shown that the IFN-I pathway is dysregulated in dermatomyositis inducing decreased myotube differentiation and endothelial dysfunction (Ladislau et al., 2018). *In vitro* study and preclinical data in four DM patients showed that ruxolitinib (a JAK1/JAK2 inhibitor) decreased IFN-I scores and improved skin manifestations (Ladislau et al., 2018). A retrospective case-control series suggested that tofacitinib may be a promising treatment in rapidly progressive interstitial lung disease in anti-MDA5 DM patients and may reduce mortality (Chen et al., 2019). A long-term extension study of a 12-week open-label trial of 10 subjects with refractory dermatomyositis treated with tofacitinib (NCT03002649) has been presented at the ACR 2020 Convergence meeting showing potential promising results. For example, the mean baseline Cutaneous Dermatomyositis Activity and Severity Index (CDASI) was 25.4 ± 15 which dropped to 3 5.43 ± 2.51 by week 68 (p = 0.01) (Paik et al., 2018; Paik et al., 2020).

Systemic sclerosis (SSc). A phase 1/2 placebo-controlled trial of tofacitinib in 15 patients with SSc was reported in the ACR 2019 meeting showing that at 6 months, skin involvement and the Combined Response Index in Systemic Sclerosis (CRISS) tended to better improvement in the tofacitinib group than controls (Khanna et al., 2019).

Other Treatments

Other treatments targeting TLRs or IFN-I downstream pathways included Syk inhibitors, Tyk2 inhibitors, TLR7, nine inhibitors, and IRF inhibitors (Chasset and Arnaud, 2018; Goess et al., 2019; Haselmayer et al., 2019; Blomgren et al., 2020; Jiang et al., 2020; Lorenzo-Vizcaya et al., 2020; Song et al., 2020), but most of these molecules are at most in early phase 2 development.

Lessons From Therapeutic Trials Targeting IFN-I in SADs

The immunopathogenic events leading to full blown SADs are obviously complex and imprecisely delineated. Nonetheless, as reviewed here by us and elsewhere by others (Hall and Rosen, 2010; Ivashkiv and Donlin, 2014; Muskardin and Niewold, 2018; Crow et al., 2019; Rönnblom and Leonard, 2019) a very large body of evidence points to a central role of IFN-I in the pathogenesis of SADs, in particular of SLE. Could the results obtained in controlled and large clinical trials provide additional information on the role of IFN-I in SADs, maybe better to say in SLE? The issue is complicated by the difficulty of capturing clinical responses in systemic disorders where multiple parameters have to be taken into account including pathogenic mechanisms, type and extent of organ involvement, concomitant use of other drugs, disease duration, and accumulated damage at time of evaluation (Thanou et al., 2014; de Jong et al., 2016; Touma and Gladman, 2017; Merrill et al., 2018). Further, it is very likely that in SLE, various pathogenic mechanisms may contribute differentially to different disease manifestations in different individuals (Banchereau et al., 2016).

The largest body of evidence currently available to address the role of IFN-I in SADs inferred by the use of inhibitors in humans has been generated in trials assessing the efficacy of anifrolumab evaluated in three trials: the MUSE phase 2b study and the phase 3 studies TULIP 1 and TULIP 2 (Furie et al., 2017; Furie et al., 2019b; Morand et al., 2020). They provided contrasting results with one of the studies (TULIP 1) not reaching its primary endpoint. One of the main differences between these trials was the choice of different primary endpoint measures (SRI-4 based on SLEDAI for MUSE and TULIP 1 and BICLA based on BILAG for TULIP 2). For the main characteristics of activity and response indexes used in SLE, *see* **Table 4**. Discrepancies observed could be explained at least in part by the fact that various elements of SLE activity are weighted differently between SLEDAI and BILAG and differentially affected by anifrolumab. In addition, BILAG index captures partial responses, whereas SLEDAI captures only complete responses, and SLEDAI but not BILAG incorporate biological parameters. Overall, taking into account five of the six primary and key secondary end points, the results favored anifrolumab over placebo suggesting clinical efficacy (Salmon and Niewold, 2019). However, the effect size remained quite low, a result which is difficult to reconcile with the potential role of IFN-I in the afferent phase of the immune response in SLE. In this respect, it is interesting to note that anifrolumab seemed to be particularly efficacious on cutaneous manifestation of SLE, and an improvement of 50% of the CLASI activity used as secondary outcome was positive in TULIP 2 and showed trends in TULIP 1 (p = 0.054) (Furie et al., 2019b; Morand et al., 2020). Moreover, a *post hoc* analysis of TULIP 1 and 2 showed that among patients with CLASI activity ≥10 (moderate to severe skin activity) (Klein et al., 2011), CLASI-A response (≥50% reduction) was achieved by week 12 in 46.0% (49/107) of patients receiving anifrolumab vs. 24.9% (24/94) receiving placebo (difference 21.0; 95% CI 8.1%, 34.0%; nominal $p < 0.001$). Moreover, time to CLASI-A response sustained to week 52 favored anifrolumab in TULIP 1 [hazard ratio (HR) 1.91; 95% CI 1.14, 3.27] and TULIP 2 (HR 1.55; 95% CI 0.87, 2.85) (Werth et al., 2020a). Thus, evidence points to a role for IFN-I in the efferent phase of the immune response, in particular in mediating skin disease in SLE. Consistently with this conclusion, alternative treatment strategies targeting IFN-I in the most advanced phase of development including baricitinib (JAKi, active phase 3) and BIIB059 (anti-BDCA2 mAB, ongoing phase 3) have shown efficacy on skin manifestations of SLE (Wallace et al., 2018; Furie et al., 2019a).

CONCLUSION

All IFNs are intimately involved in the pathogenesis of systemic autoimmunity. Most attention has been given to IFN-I. IFN-I self-stimulatory and amplificatory activity leading to high levels

of ISG in SADs may indeed profoundly affect immunopathology participating both to the immune response and to tissue damage (summarized in **Supplementary Figure S2**). Polymorphisms in gene coding for factors participating to intracellular signaling leading to IFN production or initiated by IFNs are associated with an increased risk of disease development across the diverse SADs and disease severity. Evidence however supports models in which the production of IFN-I may be preceded by other events including the production of autoAb and IFN-gamma consistently with a preceding adaptive immune response. It is also possible that PMN, or a subset of PMN, could drive or enhance the production of IFN-I. The three-partite participation of IFN-I, autoAb, and PMN cells may explain, at least in part, the chronic nature of SADs and their wax and waning course, particularly in SLE. From the therapeutic point of view, the blockade of IFN-I biological activity aiming at reducing immunopathology in SADs makes obvious sense. The inconstant results obtained up to now applying this therapeutic strategy to SLE and other SADs highlight the complexities portending autoimmunity and heterogeneity in clinical manifestations. In the era in which precision medicine is becoming a reality when addressing therapeutic approaches in oncology, we realize that understanding autoimmunity requires further investigation to subset patients with SADs in order to offer them personalized and efficacious therapies. The IFN gene signature represents an interesting biomarker to select individuals with SADs for targeted therapeutic approaches.

AUTHOR CONTRIBUTIONS

FC has conceived the structure, has reviewed the literature, and has written a large part of the manuscript. JD has critically revised the manuscript. CC has conceived the structure, has reviewed the literature, and has written a large part of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.633821/full#supplementary-material.

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Association Between Prior Calcium Channel Blocker Use and Mortality in Septic Patients: A Meta-Analysis of Cohort Studies

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Background: The aim of this study was to comprehensively review the literature and synthesize the evidence concerning the relationship between prior calcium channel blocker (CCB) use and mortality in patients with sepsis.

Methods: The Medical Literature Analysis and Retrieval System Online (MEDLINE), Excerpta Medica database (EMBASE), Cochrane CENTRAL, and Web of Science databases were searched from their inception to April 9, 2020. Cohort studies related to prior calcium channel blocker use in patients with sepsis were analyzed. Pairs of reviewers independently screened the studies, extracted the data, and assessed the risk of bias. The primary outcome of 90-days mortality or secondary outcome of short-term mortality, including 30-days, Intensive Care Unit (ICU), and in-hospital mortality, were analyzed. Heterogeneity among studies was assessed using the I^2 statistic and was considered moderate if I^2 was 50–75% and high if I^2 was ≥75%. Random-effects models were used to calculate the pooled odds ratios (ORs) and 95% confidence intervals (CIs). The quality of the studies was evaluated with the Newcastle-Ottawa Scale (NOS). Sensitivity analyses were performed to examine the robustness of the results.

Results: In total, 639 potentially relevant studies were identified, and the full texts of 25 articles were reviewed. Ultimately, five cohort studies involving 280,982 patients were confirmed to have a low risk of bias and were included. Prior CCB use was associated with a significantly lower 90-days mortality in sepsis patients [OR, 0.90 (0.85–0.95); I^2 = 31.9%]. Moreover, prior CCB use was associated with a significantly reduced short-term mortality rate in septic shock patients [OR, 0.61 (0.38–0.97); I^2 = 62.4%] but not in sepsis patients [OR, 0.83 (0.66–1.04); I^2 = 95.4%].

Abbreviations: CCB, calcium channel blocker; CIs, confidence intervals; NOS, Newcastle-Ottawa Scale; PICOS, population, intervention, comparators, outcomes and study design; LPS, lipopolysaccharide.

Conclusion: This meta-analysis suggests that prior CCB use is significantly associated with improved 90-days mortality in sepsis patients and short-term mortality in septic shock patients. This study provides preliminary evidence of an association between prior CCB use and mortality in sepsis patients.

Keywords: calcium channel blocker, sepsis, septic shock, mortality, systematic review, meta-analysis, prior

BACKGROUND

Sepsis is defined as a life-threatening disorder of organ function caused by a dysregulated host response to infection (Singer et al., 2016). Global epidemiological data suggest that sepsis is a major public health issue and remains a primary cause of mortality and critical illness; sepsis affects millions of people worldwide each year (Angus et al., 2001; Dellinger, 2003; Rhodes et al., 2017), and its incidence is not declining (Gaieski et al., 2013). Currently, the pathophysiological basis of sepsis is thought to involve disordered pro- and anti-inflammatory responses, which suggests a new method for the treatment of this deadly condition (Hotchkiss et al., 2013). The prognosis is associated with not only the virulence of the pathogens but also the septic patient's age and coexisting conditions, such cardiovascular dysfunction (Angus and van der Poll, 2013).

Calcium channel blockers (CCBs) are widely administered for the treatment of cardiovascular disease, including hypertension and ischaemic heart disease (Fihn et al., 2012; James et al., 2014). These drugs inhibit Ca^{2+} channels in the myocardium and vascular smooth muscle cells, resulting in the inhibition of myocardial contractions, the pulse conduction system (anti-arrhythmias), and vasodilation (Sueta et al., 2017). Cardiovascular disease is well known to be one of the most common coexisting conditions in septic patients and is independently related to an increased risk of death during hospitalization (Martin et al., 2003; Vincent et al., 2009). Sepsis is related to an overload of Ca^{2+} in many cell types (Hotchkiss and Karl, 1996) that can lead to disordered cellular processes, cytotoxicity or even cell death *via* a variety of mechanisms, such as metabolic manifestations, vascular smooth muscle tone dysregulation, mitochondrial dysfunction, nuclear damage, cytoskeletal breakage, the production of nitric oxide and pro-inflammatory cytokines, and apoptosis (Dong et al., 2006; Clapham, 2007). However, CCBs can restore such disrupted cellular processes to their normal states through calcium channel-dependent calcium ion homeostasis. Furthermore, CCBs exert pleiotropic effects, such as antioxidant effects (Mason et al., 1998) and immunodepression and anti-inflammatory activity suppression (Das et al., 2009), in sepsis. Therefore, CCB use may benefit patients with sepsis.

Recently, Wiewel et al. (2017) reported that previous CCB use conferred an obvious survival benefit compared with non-CCB use in patients with sepsis. However, several studies (Lee et al., 2017; Kim et al., 2019; Hsieh et al., 2020) have indicated that prior CCB use was not related to lower mortality in septic patients. In addition, Hsieh et al. (2020) reported that CCBs were associated with decreased 30-days mortality in septic shock patients. However, de Roquetaillade et al. (2020) indicated that CCB use was not associated with lower mortality in septic shock patients. Therefore, the relationship between previous CCB use and prognosis in sepsis patients is controversial. Thus, the available data were synthesized to evaluate whether CCBs are helpful for reducing mortality in septic patients.

MATERIALS AND METHODS

The study protocol is registered on the PROSPERO website (http://www.crd.york.ac.uk/PROSPERO) with the registration number CRD42019127112, and it can be found online at https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=127112.

Search Strategy

The meta-analysis of observational studies in epidemiology (MOOSE) guidelines were followed (Stroup et al., 2000). In addition, the PRISMA 2009 checklist is shown in **Supplementary Table S1**. A comprehensive literature search for cohort studies on the association between CCB use and mortality in septic patients published from the dates of database inception to April 9, 2020, was performed in the MEDLINE (www.ncbi.nlm.nih.gov/pubmed), EMBASE (www.embase.com), Cochrane CENTRAL (https://www.cochranelibrary.com/central), and Web of Science (https://apps.webofknowledge.com) databases. A combination of MeSH/Emtree terms and title, abstract or keyword terms was used. The search terms were "calcium channel blockers," "calcium channel blocking agent," "calcium antagonist," "sepsis," and "septic shock." The detailed retrieval strategy can be seen in **Supplementary Table S2**. The search was restricted to studies published in English. Furthermore, we reviewed the references of eligible articles to identify other potentially relevant studies. The literature searches were conducted independently by Xianfei Ding and Yuqing Cui.

Eligibility Criteria

Studies were considered eligible for inclusion in the meta-analysis if they met the following population, intervention, comparators, outcomes and study design (PICOS) criteria: 1) the population included adult sepsis and/or septic shock patients, 2) the intervention involved the prior use of CCBs, 3) the comparison was with no prior use of CCBs, 4) the primary outcome was 90-days mortality, or secondary outcome was short-term (30-days, Intensive Care Unit (ICU), in-hospital) mortality, 5) the study design was an observational cohort study. We excluded relevant studies that did not report the

mortality of sepsis or septic shock patients. In addition, we also excluded studies for which full texts could not be obtained and summary and review articles.

PICOS Question

Population: Adult sepsis and/or septic shock patients.
Intervention: Prior use of CCB.
Comparison: No prior use of CCB.
Outcome: Mortality.
Study design: Prospective observational or retrospective cohort studies.

Study Selection and Data Extraction

Xianfei Ding and Yuqing Cui independently screened the titles and/or abstracts of all retrieved studies to determine whether they met the eligibility criteria and noted the reason for the exclusion of each rejected article (Kappa statistic = 0.91). Key data explications were performed independently by Huoyan Liang and Lifeng Li. All disputes were settled by discussions among Dong Wang, Quancheng Kan, and Lexin Wang. The following characteristics were extracted from all the included studies: first author, year (publication), country, study design, CCB and non-CCB use in patients with sepsis, sex composition of patients, study duration, and unadjusted or adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for the primary and secondary outcomes.

Assessment of Risk of Bias

The risk of bias of the eligible studies was evaluated with the Newcastle-Ottawa Scale (NOS) for cohort studies (Wells and O'Connell, 2014). A maximum of nine points could be obtained: four points was the maximum for selection, two points was the maximum for design and analysis comparability, and three points was the maximum for the assessment of outcomes. High-quality studies received a score ≥7, whereas moderate- and low-quality studies received scores of 4–6 and ≤4, respectively. The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) criterion was used to estimate and summarize the quality of the evidence (Brozek et al., 2009).

Statistical Analysis

For binary data, we used ORs and their 95% CIs to estimate the effect sizes of our outcome of interest. The pooled ORs from the included studies were calculated with a random-effects model, and the *I-V* heterogeneity method was used to generate the forest plots. Heterogeneity (Higgins et al., 2003) among studies was evaluated by the I^2 statistic; I^2 values of 0–25% represented no heterogeneity, values of 25–50% represented slight heterogeneity, values of 50–75% represented moderate heterogeneity, and values of 75–100% represented high heterogeneity. Begg's funnel plot (Begg and Mazumdar, 1994) was constructed, and Egger's linear regression (Stuck et al., 1998) was performed to evaluate potential publication bias. Funnel plots (Hunter et al., 2014) were visually evaluated for asymmetry. One-way sensitivity analysis (Copas and Shi, 2001) was performed to evaluate the robustness of the results. All statistical analyses were performed with Stata 14.0 (College Station, TX, 77,845, United States, Serial number: 401406267051).

RESULTS

Study Selection

The initial literature search yielded 639 potentially relevant publications, and 478 records remained after removing duplicates. We then excluded 453 records after the preliminary title and abstract screening. After evaluating the full texts of the remaining 25 records, we identified five cohort studies (Lee et al., 2017; Wiewel et al., 2017; Kim et al., 2019; de Roquetaillade et al., 2020; Hsieh et al., 2020) that were eligible for inclusion in this meta-analysis (**Figure 1**).

Study Characteristics

A detailed description of the five included studies is shown in **Table 1**. In total, 280,982 septic patients were included in this meta-analysis. All the included studies were multi-centre cohort studies that involved septic patients who reported their prior use of CCBs (Lee et al., 2017; Wiewel et al., 2017; Kim et al., 2019; de Roquetaillade et al., 2020; Hsieh et al., 2020). We extracted the adjusted or propensity-matched ORs and 95% CIs from the primary and secondary outcome data. Otherwise, the data were calculated from the raw data in each included study.

Risk of Bias Assessment

The risk of bias assessment of the included studies is shown in **Table 2**. The five eligible observational cohort studies (Lee et al., 2017; Wiewel et al., 2017; Kim et al., 2019; de Roquetaillade et al., 2020; Hsieh et al., 2020) had scores ≥8 and were considered to have a low risk of bias according to the NOS.

Effects of CCB on Septic Patients

The 90-days mortality rate, which was the primary outcome, and the short-term mortality rates, which were the secondary outcomes, are shown in **Figures 2A–4A**. Prior CCB use was associated with a significantly reduced 90-days mortality rate in sepsis patients [OR, 0.90 (0.85–0.95); I^2 = 31.9%; evidence rank, very low] (**Figure 2A**). Moreover, prior CCB use was associated with a reduced short-term mortality rate in septic shock patients [OR, 0.61 (0.38–0.97); I^2 = 62.4%; evidence rank, very low] but not in sepsis patients [OR, 0.83 (0.66–1.04); I^2 = 95.4%; evidence rank, very low] (**Figures 3A**, **4A**).

Sensitivity Analysis

As the included studies were observational cohort studies with a low risk of bias (**Table 2**), a sensitivity analysis of the methodological criteria was not conducted. A sensitivity analysis was conducted to evaluate the effect of any one study on the pooled ORs and 95% CIs by removing one individual study at a time. The sensitivity analysis findings indicated that the results were robust and reliable (**Figures 2B–4B**).

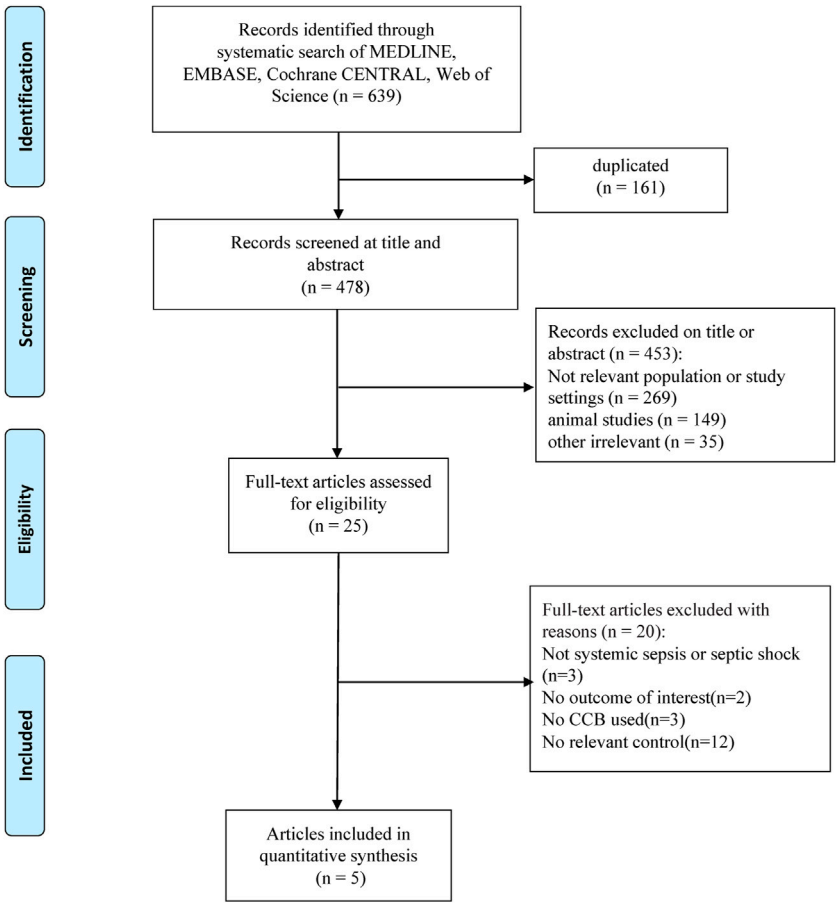

FIGURE 1 | Flow chart of the literature selection process.

Publication Bias

Because the number of included studies that reported the effects of CCB use on septic patients was small (<10), we did not generate a funnel plot, as it may not have discovered publication bias (Lau et al., 2006).

DISCUSSION

This meta-analysis involving 280,982 patients indicated that compared with no prior CCB use, prior CCB use was related to a reduced 90-days mortality rate in patients with sepsis and a reduced short-term mortality rate in patients with septic shock. To our knowledge, this is the first meta-analysis to explore and evaluate the relationship between prior CCB use and mortality in septic patients. These findings indicate that CCB administration is associated with significant improvements in the 90-days prognosis of sepsis patients and the short-term survival of septic shock patients.

Currently, the effect of prior CCB use on the mortality of septic patients remains unclear (Lee et al., 2017; Wiewel et al., 2017; Kim et al., 2019; de Roquetaillade et al., 2020; Hsieh et al., 2020). Several animal studies (Németh et al., 1998; Wyska, 2009) have suggested that CCBs could reduce mortality in endotoxaemic mouse models. Verapamil improved the survival rate of dogs with endotoxic shock (Bosson et al., 1985), and nifedipine increased survival in a bacteraemia model (Bosson et al., 1986). However, the results of clinical studies are not consistent with the results of these animal studies (Lee et al., 2017; Kim et al., 2019; Hsieh et al., 2020). This meta-analysis provides evidence supporting the association of prior CCB use with decreased mortality in patients with sepsis.

The potential mechanism underlying the association of CCB use and mortality in septic patients remains unclear. CCB may ameliorate cardiac dysfunction (Bosson et al., 1985; Zhu et al., 2005) in septic survivors with cardiovascular complications (Ou et al., 2016). A previous meta-analysis showed that prior β-blocker use was associated with a reduction in mortality, which may be due to decreased cardiac systolic and diastolic dysfunction (Tan et al., 2019). Several studies have reported that CCBs differentially inhibit the generation of pro-inflammatory factors, such as interleukin-12 (IL-12), interferon-gamma (IFN-γ) (Németh et al., 1998), and TNF-alpha (Li et al., 2009), in sepsis patients. Additionally, CCBs inhibit the nuclear transcription factor NF-κB and activate PI3K/Akt passage (Mustafa and Olson, 1999; Hayashi et al., 2000; Li et al., 2006; Hassoun et al., 2008), which reduce LPS-induced acute inflammatory reactions (Zhang et al., 2007).

TABLE 1 | Summary of identified studies.

Author/Year	Country	Study Design	Study Duration	Female/ Male	Descriptions of participants	Disease severity	Number of Patients in CCB(death)/ non-CCB Use(death)	Follow up	90-day Mortality in sepsis[a] (OR, 95% CI)	Short-term Mortality in septic shock (OR, 95% CI)	Short-term Mortality in sepsis[a] (OR, 95% CI)	Comorbidities
Wiewel et al. (2017) (18)	Netherlands	PC	2011/ 7–2013/7	420/640	Critical sepsis	SOFA 7 (5–9)/ 7 (5–9)	197(58)/863(326)	90-day	0.62 (0.40–0.96)	0.31 (0.14–0.65)	0.48 (0.31–0.74)[a]	Cerebrovascular disease Chronic cardiovascular insufficiency Chronic renal insufficiency Congestive heart failure Chronic obstructive pulmonary disease Diabetes mellitus Hematologic malignancy Hypertension Immune deficiency Metastatic malignancy Myocardial infarction Nonmetastatic malignancy Peripheral vascular disease
Lee et al. (2017) (19)	Taiwan	RC	2000–2011	20,903/ 30,175	Sepsis	Average number of organ dysfunction 1 (1–2)/1 (1–2)	19,742/31,336	90-day	0.91 (0.85–0.97)	NA	0.92 (0.85–0.99)	Myocardial infarction Congestive heart failure Peripheral vascular disease Cerebrovascular disease Dementia Chronic pulmonary disease Rheumatologic disease Peptic ulcer disease Diabetes with chronic complications Hemiplegia or paraplegia Renal disease
Kim et al. (2019) (20)	South Korea	RC	2003–2013	2,328/2,221	Sepsis	NA	1,287(586)/ 3,262(1,583)	90-day	0.89 (0.78–1.01)	NA	0.83 (0.72–0.95)	Cardiovascular Chronic respiratory disease Chronic renal disease Chronic liver disease Diabetes Cerebrovascular Solid tumor Hematologic disease
Hsieh et al. (2020) A (21)	Taiwan	RC	1999–2013	NA	Sepsis	NA	NA	28-day	NA	NA	1.21 (1.17–1.26)[b]	Hyperlipidemia Congestive heart failure chronic kidney disease chronic liver disease chronic obstructive pulmonary disease ischemic heart disease Hypertension Cancer

(Continued on following page)

TABLE 1 | (*Continued*) Summary of identified studies.

Author/Year	Country	Study Design	Study Duration	Female/ Male	Descriptions of participants	Disease severity	Number of Patients in CCB(death)/ non-CCB Use(death)	Follow up	90-day Mortality in sepsis[a] (OR, 95% CI)	Short-term Mortality in septic shock (OR, 95% CI)	Short-term Mortality in sepsis[a] (OR, 95% CI)	Comorbidities
Hsieh et al (2020) B (21)	Taiwan	RC	1999–2013	NA	Septic shock	NA	NA	28-day	NA	0.64 (0.53–0.77)	NA	
Roquetaillade et al. (2020) (22)	French	RC	2008–2016	NA	Septic shock	SOFA 9.57 (3.71)/ 10.96 (4.06)	103/632	ICU	NA	0.95 (0.52–1.74)	NA	Chronic heart failures Arterial hypertension Diabetese Obesity Cirrhosis Chronic obstructive pulmonary disease chronic kidney failure Imunosuppression

Abbreviations: PC, prospective cohort; RC, retrospective cohort; SOFA, Sequential Organ Failure Assessment; CCB, calcium channel blockers; NA, not available; OR, odds ratio; CI, confidence interval.
[a]*30-day Mortality in sepsis that included septic shock.*
[b]*30-day Mortality in sepsis that not included septic shock.*

TABLE 2 | The Newcastle-Ottawa Scale (NOS) for assessing the quality of including studies.

Studies First Author	Selection				Comparability		Assessment of outcome			Total Quality Score
	Representativeness of CCB Use Arm(s)	Selection of the non-CCB Use Arm(s)	Origin of Exposure Source	Demonstration that Outcome of Interest was not Present at Start of Study	Studies Controlling the Most Important Factors	Studies Controlling the Other Main Factors	Assessment of Outcome with Independency	Adequacy of Follow-up Length (to assess outcome)	Lost to Follow-up Acceptable (less than 10% and reported)	
Wiewel et al. (2017) (18)	*	*	*	*	*	*	*	*	*	9
Lee et al. (2017) (19)	*	*	*	*	*	*	*	*		8
Kim et al. (2019) (20)	*	*	*	*	*	*	*	*		8
Hsieh et al. (2020) (21)	*	*	*	*	*	*	*	*		8
Roquetaillade et al. (2020) (22)	*	*	*	*	*	*	*	*		8

Abbreviations: CCB calcium channel blockers. Each star represents reaching the standard, and starred items are given one point.

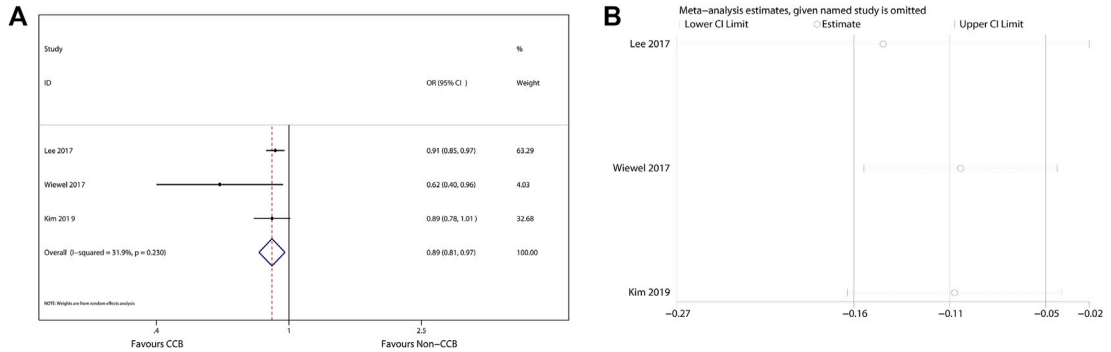

FIGURE 2 | (A) Forest plot showing the significance of the relationship between the prior use of CCBs and 90-days mortality in patients with sepsis according to the random-effects model. **(B)** The sensitivity analysis showed that the studies were robust and reliable with regard to the relationship between prior CCB use and 90-days mortality in patients with sepsis.

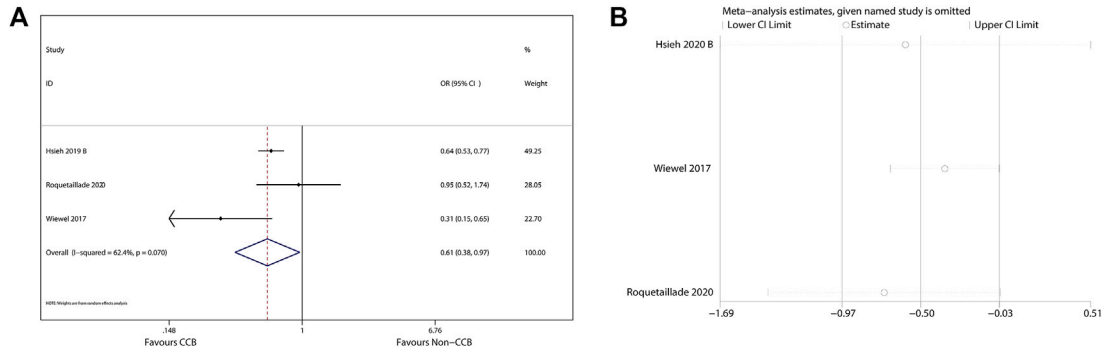

FIGURE 3 | (A) Forest plot showing the significance of the relationship between the prior use of CCBs and short-term mortality in patients with septic shock according to the random-effects model. **(B)** The sensitivity analysis showed that the studies were robust and reliable for the association of prior CCB use with short-term mortality in patients with septic shock.

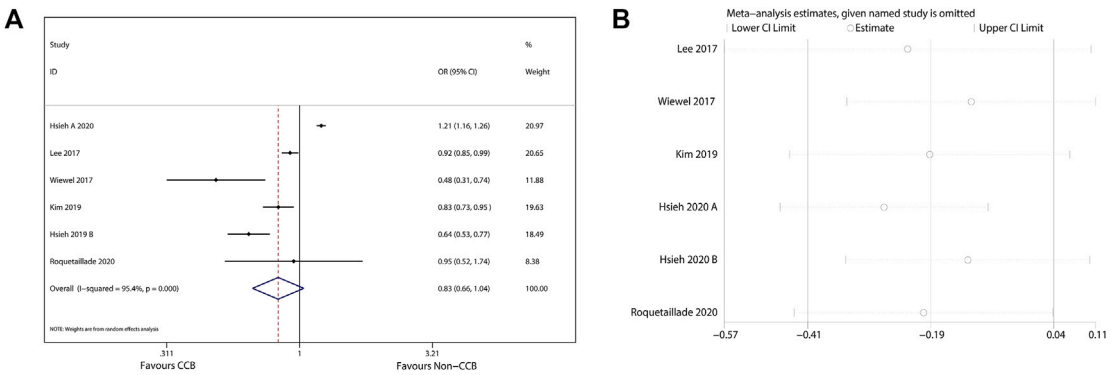

FIGURE 4 | (A) Forest plot showing the significance of the relationship between the prior use of CCBs and short-term mortality in patients with sepsis according to the random-effects model. **(B)** The sensitivity analysis showed that the studies were robust and reliable with regard to the relationship between prior CCB use and short-term mortality in patients with sepsis.

Moreover, CCBs have been shown to lower oxidative burst and inducible nitric oxide synthase (iNOS) protein expression to regulate the inflammatory response (Hotchkiss et al., 1997) and ameliorate cellular injury and cardiac dysfunction. Most importantly, sepsis disrupts intracellular calcium homeostasis, which leads to endothelial injury and destroys subcellular

structures (Duchen, 2000; Ding et al., 2013). CCBs, which are involved in targeting and blocking calcium ion overload (Meldrum et al., 1993; Song et al., 1993), could reduce intracellular Ca^{2+} levels and prevent cytotoxicity. However, the relationship between CCB administration and an improved prognosis of sepsis needs to be confirmed in clinical trials.

A meta-analysis is used to systematically and statistically analyze a variety of studies on the same topic. The summarized meta-analysis results have significant heterogeneity when the differences among outcomes in the included individual studies are greater than expected. In the present meta-analysis, the assessment of the risk of bias in the included studies showed a low risk of bias; thus, methodological heterogeneity did not exist.

This meta-analysis has a number of advantages. First, the sample of included septic patients was large, suggesting that the results may be stable. The large population was sufficient to conduct propensity score matching, which is a method of reducing the effects of deviations and confounding variables between the CCB and the non-CCB groups. Second, the NOS was used to assess the risk of bias. The results indicated that the studies that met the inclusion criteria for this meta-analysis had a low risk of bias. Third, we extracted the adjusted or propensity-score matched ORs and 95% CIs to calculate the pooled ORs for the effect of CCB use on mortality in an unbiased manner. Fourth, the sensitivity analysis suggested that the results were robust and reliable.

However, this meta-analysis has several limitations. Although we conducted an overall search to identify the pertinent studies as far back as possible, only five studies were included; more research may be needed to confirm this conclusion. Nevertheless, the robustness of the conclusion was supported by the sensitivity analysis. In addition, our study was limited by high heterogeneity. According to the inclusion criteria for each study, there were differences in racial and other characteristics of the participants and the timing and use of antihypertensive drugs, leading to high heterogeneity. In addition, the studies included in this meta-analysis were only observational studies, not randomized controlled trials. A certain limitation exists even if all the included cohort studies show a low risk of bias. The effectiveness of prior CCB treatment on mortality in sepsis and septic shock patients needs to be further investigated in high-quality studies.

CONCLUSION

This is the first systematic review and meta-analysis to report the association between prior CCB use and mortality in septic patients. We discussed the effects of prior CCB use on cardiovascular function and inflammation in sepsis. This meta-analysis suggests that prior CCB use is significantly associated with improvements in the 90-days prognosis of sepsis patients and the short-term survival of septic shock patients. However, this finding should be evaluated in future randomized controlled trials. CCBs remain an attractive potential method for the improvement of sepsis-related mortality.

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 author.

AUTHOR CONTRIBUTIONS

All the authors contributed substantially to the work presented in this article. TS, XD, and YC conceived of the study. HL and LL contributed to the data interpretation. XD and YC contributed to the study protocol and wrote the article. DW, QK, and LW settled controversies. QK, LW, and TS revised the article. All authors approved the final version submitted for publication. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

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Histamine H_3 Receptor Signaling Regulates the NLRP3 Inflammasome Activation in C2C12 Myocyte During Myogenic Differentiation

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NLRP3 inflammasome has been implicated in impaired post-injury muscle healing and in muscle atrophy. Histamine receptors play an important role in inflammation, but the role of histamine H_3 receptor (H_3R) in myocyte regeneration and in the regulation of NLRP3 inflammasome is not known. We studied the effects of H_3R signaling on C2C12 myocyte viability, apoptosis, and tumor necrosis factor alpha (TNFα)-induced NLRP3 inflammasome activation during striated myogenic differentiation at three time points (days 0, 3, and 6). Expression of *Nlrp3*, interleukin-1β (IL-1β), and myogenesis markers were determined. TNFα reduced overall viability of C2C12 cells, and exposure to TNFα induced apoptosis of cells at D6. Activation of H_3R had no effect on viability or apoptosis, whereas inhibition of H_3R increased TNFα-induced apoptosis. Stimulation of C2C12 cells with TNFα increased *Nlrp3* mRNA expression at D3 and D6. Moreover, TNFα reduced the expression of myogenesis markers MyoD1, Myogenin, and Myosin-2 at D3 and D6. H_3R attenuated TNFα-induced expression of *Nlrp3* and further inhibited the myogenesis marker expression; while H_3R -blockage enhanced the proinflammatory effects of TNFα and increased the myogenesis marker expression. TNFα-induced secretion of mature IL-1β was dependent on the activation of the NLRP3 inflammasome, as shown by the reduced secretion of mature IL-1β upon treatment of the cells with the small molecule inhibitor of the NLRP3 inflammasome (MCC950). The activation of H_3R reduced TNFα-induced IL-1β secretion, while the H_3R blockage had an opposite effect. In conclusion, the modulation of H_3R activity regulates the effects of TNFα on C2C12 myocyte differentiation and TNFα-induced activation of NLRP3 inflammasome. Thus, H_3R signaling may represent a novel target for limiting postinjury muscle inflammation and muscle atrophy.

Keywords: histamine H_3 receptor, myogenesis, inflammation, NLRP3 inflammasome, IL-1β, TNFα, C2C12 myocyte

INTRODUCTION

Inflammation can complicate post-injury muscle healing and slow down the regenerative repair process (Hofer et al., 2014). However, the factors underlying the post injury inflammation are poorly known. It is believed that activation of the inflammasomes, with ensuing production of highly proinflammatory cytokines, IL-1β and IL-18, may play a key role by initiating the inflammatory reaction (Mangan et al., 2018). Nucleotide-binding domain and leucine-rich repeat containing family (NLR), pyrin domain containing 3 (NLRP3) inflammasome is the most widely studied inflammasome. It is activated by a myriad of factors, including exogenous pathogens and endogenous danger signals, and has been implicated in a variety of immunological (Mulay et al., 2018) and non-immunological diseases (Hughes et al., 2016). The NLRP3 inflammasome is a multi-protein complex, consisting of the sensor protein NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and the precursor of caspase 1 (pro-caspase-1). Upon activation, NLRP3 is associated with pro-caspase-1 via the adaptor protein ASC resulting in autoactivation of pro-caspase-1 into its active form. The active caspase-1 then catalyzes the cleavage of the inactive precursors of interleukin 1β (pro-IL-1β) and interleukin 18 (pro-IL-18) into their active secreted forms, thus triggering the inflammatory storm (Man and Kanneganti, 2015). NLRP3 inflammasome is mainly expressed in innate immune cells such as neutrophils, macrophages, monocytes, and dendritic cells, but low expression levels are seen in other cell types and tissues (Latz et al., 2013; Elliott and Sutterwala, 2015; Guo et al., 2015; Jo et al., 2016). NLRP3 has been shown to be expressed also in myocytes (Wei et al., 2020) and it has been implicated in the pathogenesis of inflammatory myopathies (Boursereau et al., 2018).

After exercise, skeletal muscle can secrete many bioactive molecules, known as myokines, and tumor necrosis factor alpha (TNFα) is one of the most common exercise-regulated myokines (also named adipo-myokines) (Görgens et al., 2015), which can induce autophagy and apoptosis in mouse C2C12 myoblasts and myotubes (Gallo et al., 2015). The inflammation-associated insulin resistance of C2C12 myoblasts and myotubes has been suggested to be mediated by the activation of NLRP3 inflammasome (Cho and Kang, 2015).

There are four known histamine receptors, of which histamine H_3 receptor (H_3R) is mainly expressed in immune and nerve cells, and is involved in nerve conduction, muscle contraction, gastrointestinal neuromodulation, and inflammation (Branco et al., 2018). We found previously that H_3R is expressed in C2C12 myoblasts and in primary adult mid-urethral striated muscles (Chen et al., 2015; Chen et al., 2015). In C2C12 myoblasts the expression of H_3R was increased during their myogenic differentiation into striated myocytes, and in myocytes the activation of the H_3R facilitated the relaxation of the cells by limiting the cytoplasmic calcium peak (Chen et al., 2015). Histamine and histamine receptors possess immunomodulatory effects (Branco et al., 2018). Histamine has been reported to dampen the inflammatory effects of lipopolysaccharides, and in particular, histamine has been shown to inhibit the secretion of IL-1β in microglial cells (Barata-Antunes et al., 2017). In present study we explore further the role of H_3R in regulation of myoblast differentiation and myocyte function. We hypothesized that H_3R could modulate the secretion of IL-1β by regulating the activation of the NLRP3 inflammasome. By utilizing highly selective H_3R agonists (methimepip, Met) (Chen et al., 2015; Panula et al., 2015), and H_3R blocker (ciproxifan, CPX) (Chen et al., 2015; Panula et al., 2015) we show that activation of H_3R signaling mitigates the TNFα-induced NLRP3 inflammasome activation, and thus H_3R signaling could represent a potential target for treatment in conditions involving muscle inflammation and inflammation induced muscle atrophy.

EXPERIMENTAL

Materials

The cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was purchased from Biowhittaker/Lonza, penicillin-streptomycin from Cambrex/Lonza, Glutamine [Gibco® GlutaMAX™ Supplement (35050061)], and heat inactivated fetal bovine serum (FBS, 10100147) was from Thermo Fisher Scientific. The primers were designed and purchased from Sangon Biotech, China. Mouse IL-1β enzyme-linked immunosorbent assay (ELISA) Kit (D720335-0096) and recombinant murine TNFα (C600052-0005) were purchased from Sangon Biotech, China. The Cell Counting Kit (CCK-8) (C0038) and Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (C1063) were purchased from Beyotime Corporation, China. H_3R agonist methimepip (Met) (sc-204080) was from Santa Cruz Corporation, and ciproxifan (CPX) (C6492), H_3R blocker, was from SIGMA Inc. MCC950 (CP-456773, 210826-40-7, AbMole, China) was purchased from AbMole Inc.

Cell Culture

Mouse C2C12 myoblasts were obtained from American Type Culture Collection, and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO #15140-122), and glutamine at 37°C in humidified 5% CO_2-in-air. In the differentiation medium, FBS was reduced from 10 to 1%, but otherwise the differentiation medium had the same composition as the growth medium. The cells were passaged at 80% confluence by trypsinization (0.5% trypsin in 0.5 mM EDTA, Gibco BRL, Life Technologies, Gaithersburg, MD).

C2C12 Cell Stimulation

C2C12 cells were seeded in 96-well plates (1.6×10^4 cells/well), 12-well plates (5×10^4 cells/well), or six-well plates (4×10^5/well) in the growth media and cultured for three days before changing to differentiation media. The differentiation time points are calculated as follows: D0 represent the cells that were collected 3 days after seeding and have not received differentiation media; D3 cells received differentiation media 3 days after seeding, and were cultured for 3 days in differentiation media; D6 received differentiation media 6 days after seeding, and were cultured for 6 days in differentiation media. Collected samples were subjected on analysis of proliferation and viability (CKK-8 assay), apoptosis and necrosis (AnnexinV/PI assay) and gene expression (qPCR).

The experimental protocol is described as a flowchart in **Supplementary Figure S1A**.

Experimental groups 1–4 represent the non-differentiated myocytes, that were collected on differentiation day 0 (D0) 12 h after stimulations with TNFα, TNFα+ Met, TNFα+ CPX, or left unstimulated.

Groups 5–8 were cultured for 3 days (D3) in differentiation media, after which they were stimulated for 12 h with TNFα, TNFα+ Met, TNFα+ CPX, or left unstimulated (Differentiation media was changed on D1 and D2).

Groups 9–12 represent the fully differentiated myocytes that were cultured for 6 days in differentiation media. D6 cells were collected after 12 h stimulations with TNFα, TNFα+ Met, TNFα+ CPX, or left unstimulated (Differentiation media was changed on D3, D4, and D5).

All the above-mentioned treatments were applied on cells with identical treatments with or without 1 h preincubation of the cells in the presence of MCC950 (1 μM), which was left on the cells during stimulations. Experiment media were collected for ELISA, and cell lysates were subjected to western blot analysis (**Supplementary Figure S1B**). Simultaneously cultured cells with Met or CPX alone were analyzed for apoptosis by flow cytometry (FCM).

Cell Count Kit-8 (CCK-8) Assay

The cell count kit-8 (CCK-8), a rapid and highly sensitive detection kit for cell proliferation and cytotoxicity, was used to analyze the cell viability according to the manufacturer's protocol (Beyotime Corporation, Shanghai, China).

Apoptosis Detection

Cell apoptosis was detected with FCM. AnnexinV/PI apoptosis detection kit was used according to the manufacturer's protocols (Beyotime Corporation, Shanghai, China). The method is based on the cytofluorimetric assay established by Vermes et al. The kit discriminates intact cells (Annexin-/PI-) from apoptotic (Annexin +/PI−) and necrotic cells (Annexin +/PI+) (Vermes et al., 1995).

Quantitative Real-Time RT-PCR Analysis

C2C12 cells were harvested and their total cellular RNA was purified using RNeasy Mini Kit (QIAGEN, Düsseldorf, Germany) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA). Quantitative RT-PCR (qPCR) was performed with 100 ng first-strand cDNA using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The copy numbers of mRNA in the samples analyzed were determined in triplicate and normalized against PBGD gene as an endogenous control and the relative units were calculated using the comparative Ct method. The following primers were designed with the NCBI Primer Blast program:

Il1b sense primer (F, forward): 5′-GCAACTGTTCCTGAACTC AACT-3′, anti-sense primer (R, reverse): 5′-ATCTTTTGGGGT CCGTCAACT-3′; *Nlrp3* sense primer (F, forward): 5′-ATTACC CGCCCGAGAAAGG-3′, anti-sense primer (R, reverse): 5′-TCG CAGCAAAGATCCACACAG-3′; *MyoD1* sense primer (F, forward): 5′-CCACTCCGGGACATAGACTTG-3′, anti-sense primer (R, reverse): 5′-AAAAGCGCAGGTCTGGTGAG-3′; Myogenin sense primer (F, forward): 5′-GAGACATCCCCCTAT TTCTACCA-3′, anti-sense primer (R, reverse): 5′-GCTCAGTCC GCTCATAGCC-3′; *Myosin-2* sense primer (F, forward): 5′-GTC AGCACCATGTCTTATGGG-3′, anti-sense primer (R, reverse): 5′-TTTGCCAAATCGGGAAGAGTT-3′; *PBGD* sense primer (F, forward): 5′-AGGTCGGTGTGAACGGATTTG-3′, anti-sense primer (R, reverse): 5′-GGGGTCGTTGATGGCAACA-3′.

Western Blotting

Cells were lyzed in 150 μl of Mammal Cell Protein Extraction Reagent (CWBiotech, Beijing, China). The total protein concentration was determined with a Pierce BCA protein assay kit (Trans, Beijing, China). For each sample 40 μg of protein was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis consisting of 5% stacking gel and 12% resolving gel, and the proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST). The membranes were incubated overnight at 4 °C with mouse monoclonal antibodies against IL-1β (3A6, mouse mAb #12242S, Cell Signaling Technology, Shanghai, China) in TBST with 5% nonfat milk. The secondary antibody goat anti-mouse IgG, HRP conjugated (CW0102S, CWBiotech, Beijing, China) was used to incubate the membrane for 2 h at room temperature (RT). Labeling was performed using an enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific, Thermo, United States). The density and area of the bands was quantitated.

ELISA Analyses

C2C12 cells were incubated in the presence of TNFα, with or without Met or TNFα or MCC950 at day D0, D3, D6 for 24 h. Mouse IL-1β concentrations in the cell culture media were analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocols (Sangon Biotech, Shanghai, China).

Statistical Analysis

Calculations were performed using GraphPad InStat3 for Macintosh software. Overall significance level between the stimulated and control groups or between stimulated and inhibited groups was analyzed using one-way ANOVA, with the Dunnett and Tukey-Kramer multiple comparisons tests when appropriate. Statistical significance was set at $p < 0.05$. The data are calculated as mean ± SEM of 12 samples (three repeated experiments, each treatment performed in four replicates).

RESULTS

Effects of TNFa, and Modulation of H_3R Signaling on Myocyte Viability

The C2C12 cells were fully differentiated at D6 in each stimulation (**Figure 1A**). During C2C12 myogenesis, TNFα decreased cell viability at all the measured time points, days 0, 3, 6 (D0, D3, D6), by 20.5% ($p < 0.05$), 29.8% ($p < 0.05$), and 29.7% ($p < 0.05$),

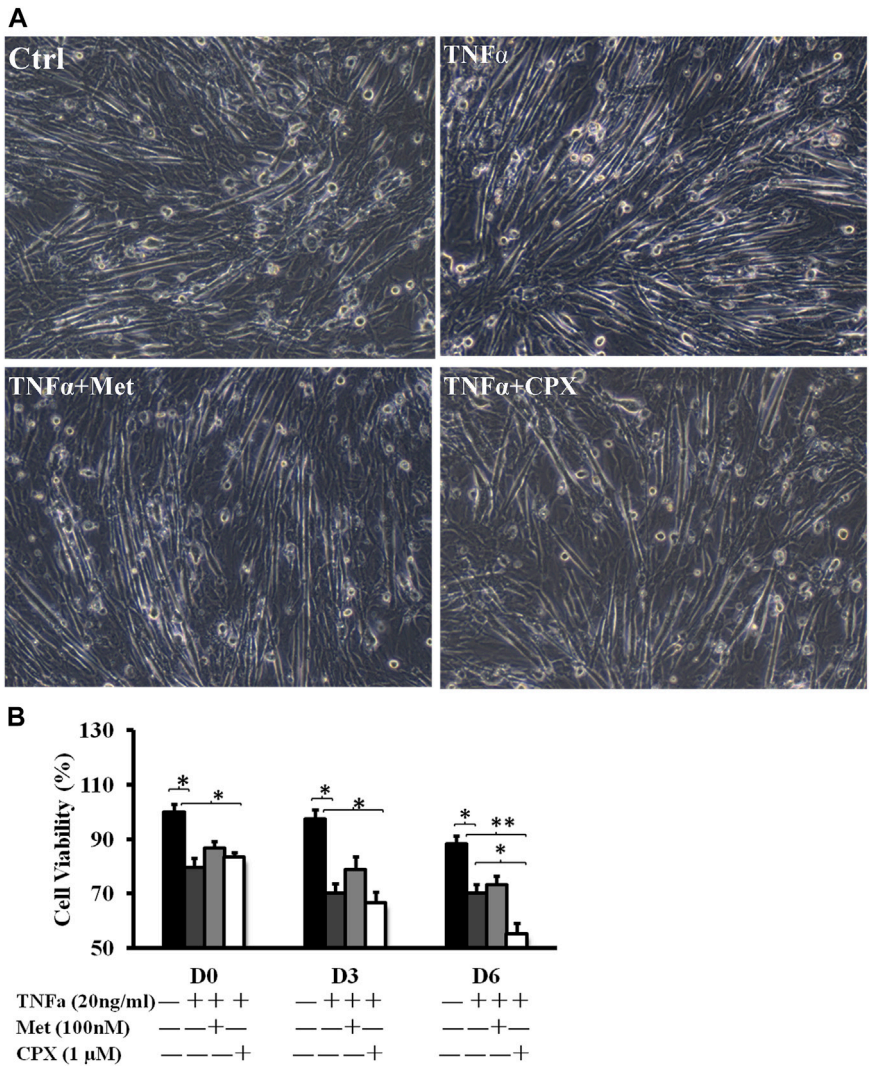

FIGURE 1 | The effect of TNFα, Met and CXP on cell viability. **(A)**: D6 C2C12 cells incubated in the presence of TNFa (20 ng/ml), TNFa + Met (100 nM) or TNFa + CPX (1 μM) for 12 h and observed by phase contrast microscopy (100×). **(B)**: Viability at D0, D3, and D6. C2C12 cells were incubated for 12 h in the presence of TNFa, TNFa + Met or TNFa + CPX. The cell death was detected by cell count kit-8 assay; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control of the same differentiation day (black columns); normalizing D0 ctrl = 100%. There was no statistical difference when TNFa + Met or TNFa + CPX was compared with TNFa-treatment of 0- and 3-differentiation day (dark gray columns). Ctrl, control; TNFa, murine Tumor Necrosis Factor-alpha; Met, methimepip; CPX, ciproxifan. D0, undifferentiated cells; D3, cells differentiated for 3 days; D6, cells differentiated for 6 days.

respectively, as compared to the controls. Addition of H_3R antagonist ciproxifan (CPX) together with TNFα had no additional effect on cell viability at the time points D0 and D3, but further reduced the viability at time point D6 (40.3%, $p < 0.05$). Meanwhile, addition of H_3R agonist methimepip (Met) together with TNFα had no additional effect on cell viability at any of the time points of D0, D3, and D6 (**Figure 1**).

Inhibition of H_3R Signaling Potentiates TNFα-Induced Apoptosis

Met alone did not induce apoptosis, however, 15.3% of the cells stimulated with CPX were apoptotic ($p < 0.05$) at D6 (**Figures 2A,B**). Next, we studied the effect of H_3R inhibition on TNFα-induced apoptosis. During C2C12 myogenesis, apoptosis and necrosis were detected in 4–6.9% of the control cells. Compared to the control cells, TNFα or TNFα +Met did not cause an obvious increase in apoptosis or necrosis during myogenic differentiation (**Figure 3**). However, treatment with combination of TNFα +CXP induced overall 61.3% ($p < 0.01$) apoptotic cells on D6, among which early apoptosis was observed in 29.9% ($T = 4.2$, $p < 0.05$) of the cells and late apoptosis in 31.4% of the cells ($T = 5.0$, $p < 0.01$) (**Figure 3**).

Expression of NLRP3 Receptor mRNA and Myogenesis Markers

As previous study has shown that another proinflammatory cytokine, IL-1β, induces the expression of *Nlrp3* in C2C12 cells (Huang et al.,

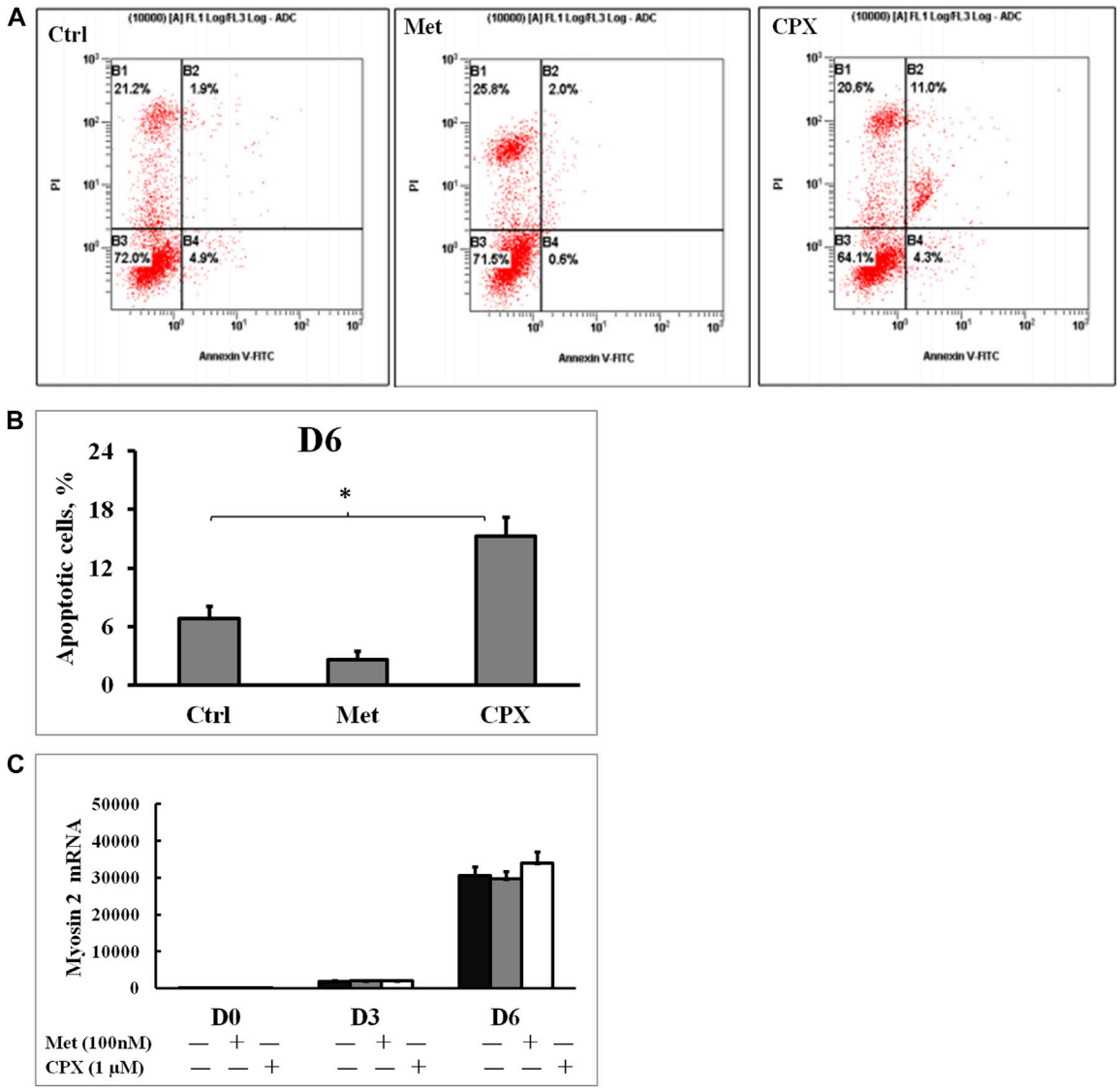

FIGURE 2 | The effect of histamine H3 receptor modulators on cell apoptosis and myogenesis. **(A)**: Apoptotic cells were detected by flow cytometry using Annexin V and propidium iodide staining; percentage of mechanically damaged cells **(top left quadrant)**, live cells **(bottom left)**, early apoptotic cells **(bottom right)**, late apoptotic cells **(top right)**. **(B)**: Percentage of apoptotic cells was calculated by adding the early and the late apoptotic cells for each experimental condition.*$p < 0.05$ vs. control. **(C)**: The C2C12 myocytes were incubated in the presence of Met (100 nM) or CPX (1 μM) for 12 h at D6, and the effect of H3 receptor inhibition or activation on myogenesis was assessed by analyzing the expression of the late myogenesis marker, myosin 2, mRNA by real time RT-PCR.*$p < 0.05$ vs. control of the same differentiation day (black columns); normalizing D0 ctrl=1. There was no statistical difference when Met or CPX was compared with control group (dark gray columns). Ctrl, control; Met, methimepip; CPX, ciproxifan. D0, undifferentiated cells; D3, cells differentiated for 3 days; D6, cells differentiated for 6 days.

2017), we studied the involvement of the NLRP3 inflammasome in the TNFα-induced inflammation. We analyzed the *Nlrp3* expression during C2C12 myogenesis on D0, D3 and D6. TNFα induced the expression of *Nlrp3* mRNA up to 201% at D3 ($p < 0.01$) and 190% at D6 ($p < 0.01$) as compared to the controls (**Figure 4A**). Met reduced the TNFα-induced expression of *Nlrp3* by 33% at D3 ($p < 0.05$) and 30% at D6 ($p < 0.05$), while CPX enhanced the effects of TNFα on *Nlrp3* expression by 24% ($p < 0.05$) at D6 (**Figure 4A**).

To study the effects of TNFα and H_3R modulation in myogenic differentiation, we analyzed the expression of the conventional myogenic markers. Myogenic Differentiation 1 (MyoD1) is considered as one of the earliest myogenic markers. MyoD1 is a nuclear protein that belongs to the subfamily of myogenic factors, and it regulates muscle cell differentiation by inducing cell cycle arrest, which is a prerequisite for myogenic initiation (Blais et al., 2005). Myogenin is usually regarded as an early myogenic marker. It is a muscle-specific transcription factor that is involved in coordination of skeletal muscle development or myogenesis and repair, and it is essential for the development of

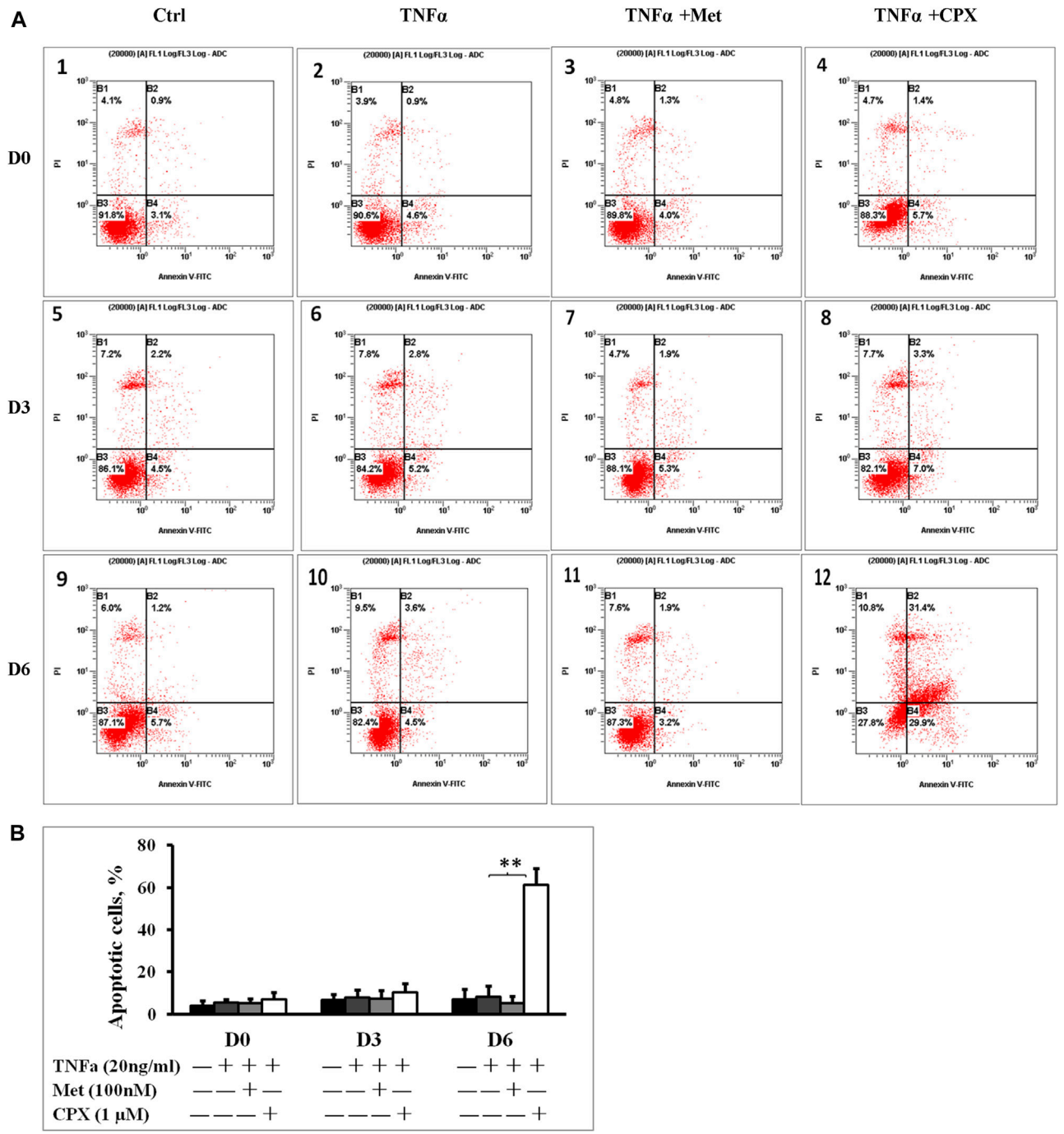

FIGURE 3 | Inhibition of histamine H_3 receptor enhances apoptosis. **(A)**: C2C12 cells were incubated in the presence of TNFa (20 ng/ml), TNFa + Met (100 nM) or TNFa + CPX (1 μM) at D0, D3, or D6. Apoptotic cells were detected by Annexin V and propidium iodide staining and analyzed by flow cytometry: percentage of cells mechanically damaged **(top left quadrant)**, live cells **(bottom left)**, early apoptotic cells **(bottom right)**, late apoptotic cells **(top right)**. **(B)**: Percentage of apoptotic cells was calculated by adding the early and the late apoptotic cells for each experimental condition. $^{**}p < 0.01$ vs. TNFα of the same differentiation day (dark gray columns). Ctrl, control; TNFa, murine Tumor Necrosis Factor-alpha; Met, methimepip; CPX, ciproxifan. D0, undifferentiated cells; D3, cells differentiated for 3 days; D6, cells differentiated for 6 days.

functional skeletal muscle (Blais et al., 2005). Myosin heavy chain 2 (Myosin-2) is a late myogenic marker. It is a member of the class II or conventional myosin heavy chains, and functions in skeletal muscle contraction (Stuart et al., 2016). TNFα reduced the expression of myogenesis markers: MyoD1 at D3 and D6 by 38% ($p < 0.05$) and 22% ($p < 0.05$) (**Figure 4C**), Myogenin at D3

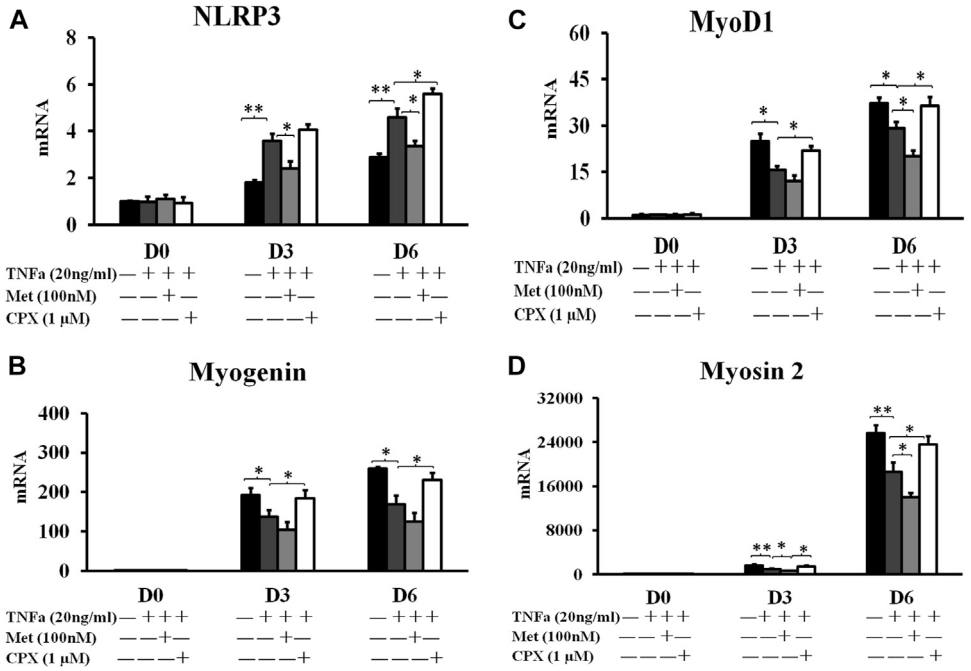

FIGURE 4 | The mRNA expression of inflammasome NLRP3 protein and myogenesis markers. C2C12 cells were incubated in the presence of TNFa (20 ng/ml), TNFa + Met (100 nM) or TNFa + CPX (1 μM) for 12 h at D0, D3, or D6. The mRNA expressions were assessed by real time RT-PCR. *$p < 0.05$, and **$p < 0.01$ between two compared groups. Normalizing D0 ctrl = 1 after compared with housekeeping gene PBGD. **(A)**: NLRP3 mRNA expression. **(B)** The mRNA expression of early myogenesis marker, MyoD1. **(C)** The mRNA expression of intermediate myogenesis marker, Myogenin. **(D)** The mRNA expression of late myogenesis marker, myosin 2. Ctrl, control; TNFa, murine Tumor Necrosis Factor-alpha; Met, methimepip; CPX, ciproxifan. D0, undifferentiated cells; D3, cells differentiated for 3 days; D6, cells differentiated for 6 days. NLRP3, Nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing three.

and D6 by 29% ($p < 0.05$) vs. 35% ($p < 0.05$) (**Figure 4B**), and Myosin-2 at D3 and D6 by 42% ($p < 0.01$) and 28% ($p < 0.01$) (**Figure 4D**), respectively. Met enhanced the inhibitory effect of TNFα of the above three markers by 23 and 31% ($p < 0.05$), 22 and 26%, 30% ($p < 0.05$) and 25% ($p < 0.05$) at D3 and D6, respectively, while CPX reduced the inhibitory effect of TNFα on the myogenic marker expression by 40% ($p < 0.05$) and 25%, 35% ($p < 0.05$) and 37% ($p < 0.05$), 50% ($p < 0.05$) and 27% ($p < 0.05$) (**Figures 4B–D**) respectively. Neither Met nor CPX alone had a clear effect on the mRNA expression of the late myocyte differentiation marker, myosin 2 ($p > 0.05$) (**Figure 2C**).

H_3R Activation Regulates the Levels of Mature IL-1β in TNFα-Treated Cells

To study the effects of TNFα and H_3R modulation on the protein expression IL-1β, we detected the levels of pro-IL-1β (31 kDa) and IL-1β (17 kDa) proteins from cell lysates by western blot. TNFα increased the levels of both the pro-IL-1β (31 kDa) and IL-1β (17 kDa) proteins ($p < 0.05$), and neither Met nor CPX had an effect on TNFα-induced pro-IL-1β expression in D0, D3 and D6 cells ($p > 0.05$). However, Met and CPX modulated the TNFα-induced expression of mature IL-1β protein; Met reduced the level of the mature IL-1β at all the time points ($p < 0.05$) in TNFα-induced D0 and D6 cells, while CPX increased it ($p < 0.05$) at D0, and D6 time points. MCC950, a new specific small molecule NLRP3 inhibitor, reduced the pro-IL-1β (31 kDa) and mature IL-1β (17 kDa) protein levels in each treatment group, except for control group ($p < 0.05$) (**Figure 5**).

H_3R Signaling Modulates the Secretion of IL-1β

Since the secretion of mature IL-1β is a marker of inflammasome activation, we next assessed the effect of TNFα and H_3R modulation on the inflammasome activation by analyzing IL-1β secretion during C2C12 myogenesis. TNFα increased the secretion of IL-1β at D0, D3 and D6 cells to 56.8 ($p < 0.01$), 66.8 ($p < 0.01$), and 85.8 pg/ml ($p < 0.01$), respectively. Met reduced the TNFα-induced secretion of IL-1β to 38.4 ($p < 0.05$), 48.4 ($p < 0.05$), 65.8 ($p < 0.05$) pg/ml, while CPX enhanced the TNFα-induced secretion of IL-1β to 67.5 ($p < 0.05$), 77.5 ($p < 0.05$), 98.9 ($p < 0.05$) pg/ml on D0, D3 and D6 cells, respectively (**Figure 6**). MCC950 reduced TNFα-induced IL-1β secretion to 41.2 ($p < 0.05$), 56.8 ($p < 0.05$), and 66.8 pg/ml ($p < 0.05$); and MCC950 reduced Met-TNFα-induced IL-1β secretion to 31.9 ($p < 0.05$), 38.4 ($p < 0.05$), 48.4 ($p < 0.05$) pg/ml. MCC950 reduced also CPX-TNFα-induced IL-1β secretion to 49.8 ($p < 0.05$), 67.5 ($p < 0.05$), 77.5 ($p < 0.05$) pg/ml (**Figure 6**). Thus, our results suggest that H_3R plays a role in regulation of NLRP3 inflammasome. H_3R inhibition reduces the TNFα-induced secretion of IL-1β whereas H_3R activation increases IL-1β secretion in C2C12 myocytes. The secretion of IL-1β was dependent on the NLRP3 inflammasome, as the NLRP3 inhibitor, MCC950, reduced TNFα induced IL-1β secretion in each groups of D0, D3 and D6 cells.

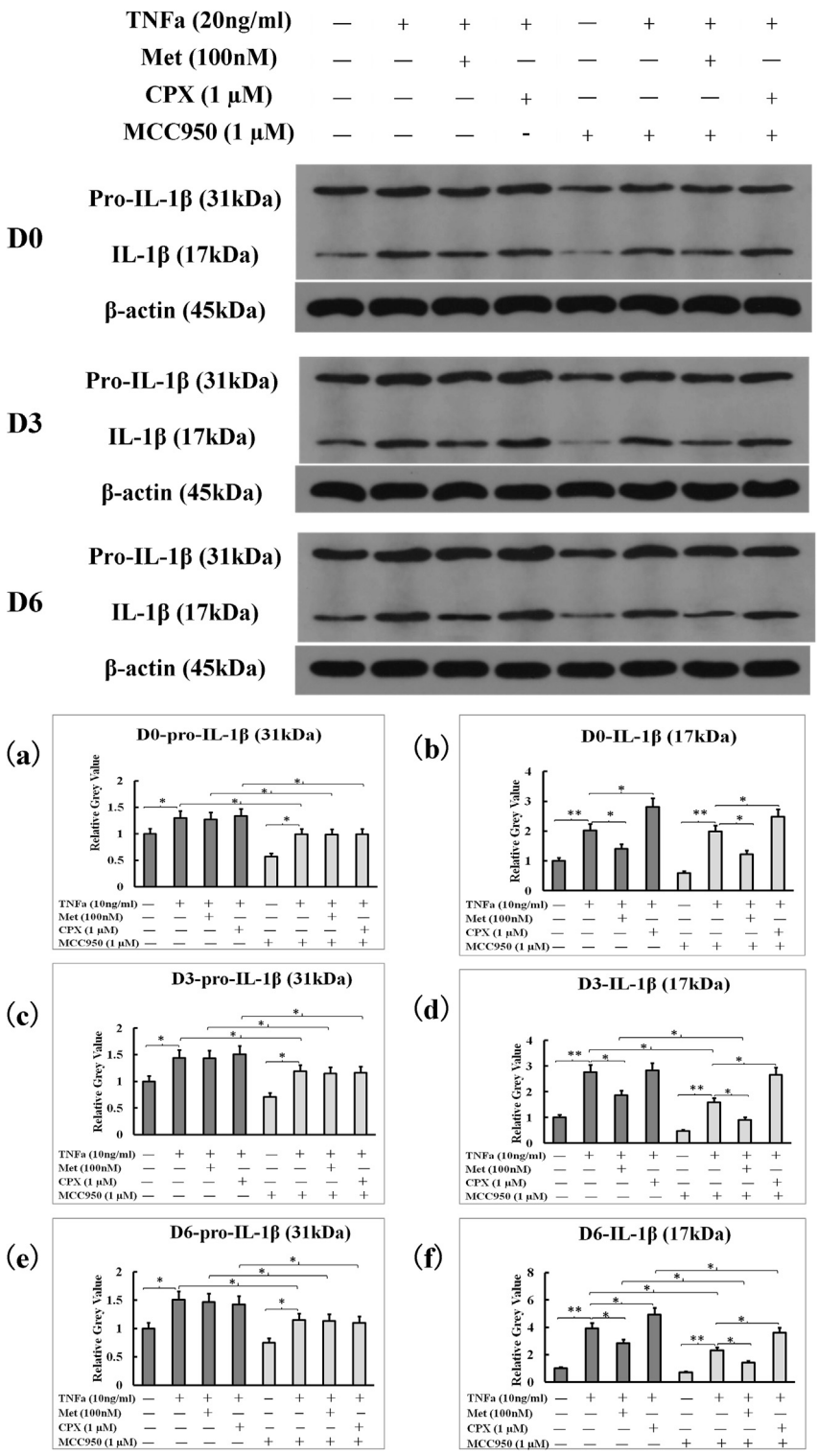

FIGURE 5 | NLRP3 inhibitor, MCC950, inhibited overall pro-IL-1β and IL-1β protein levels induced by TNFα. C2C12 cells were incubated in the presence of TNFa (20 ng/ml), TNFa + Met (100 nM) or TNFa + CPX (1 μM) or overall MCC950 (1 μM) for 12 h at D0, D3, or D6. The pro-IL-1β (31 kDa) and mature IL-1β protein (17 kDa) levels were assessed by westernblot. $^{*}p < 0.05$, and $^{**}p < 0.01$ between two compared groups. **(A,B)**: The grey-values of westernblot on pro-IL-1β and IL-1β proteins at D0 were calculated by normalized protein expressions to β-actin. **(C,D)**: The grey-values of westernblot on pro-IL-1β and IL-1β proteins at D3 were calculated by normalized protein expressions to β-actin. **(E,F)**: The grey-values of westernblot on pro-IL-1β and IL-1β proteins at D6 were calculated by normalized protein expressions to β-actin. Ctrl, control; TNFa, murine Tumor Necrosis Factor-alpha; Met, methimepip; pro-IL-1β, the precursor of interleukin 1beta; IL-1β, interleukin 1beta; CPX, ciproxifan. D0, undifferentiated cells; D3, cells differentiated for 3 days; D6, cells differentiated for 6 days.

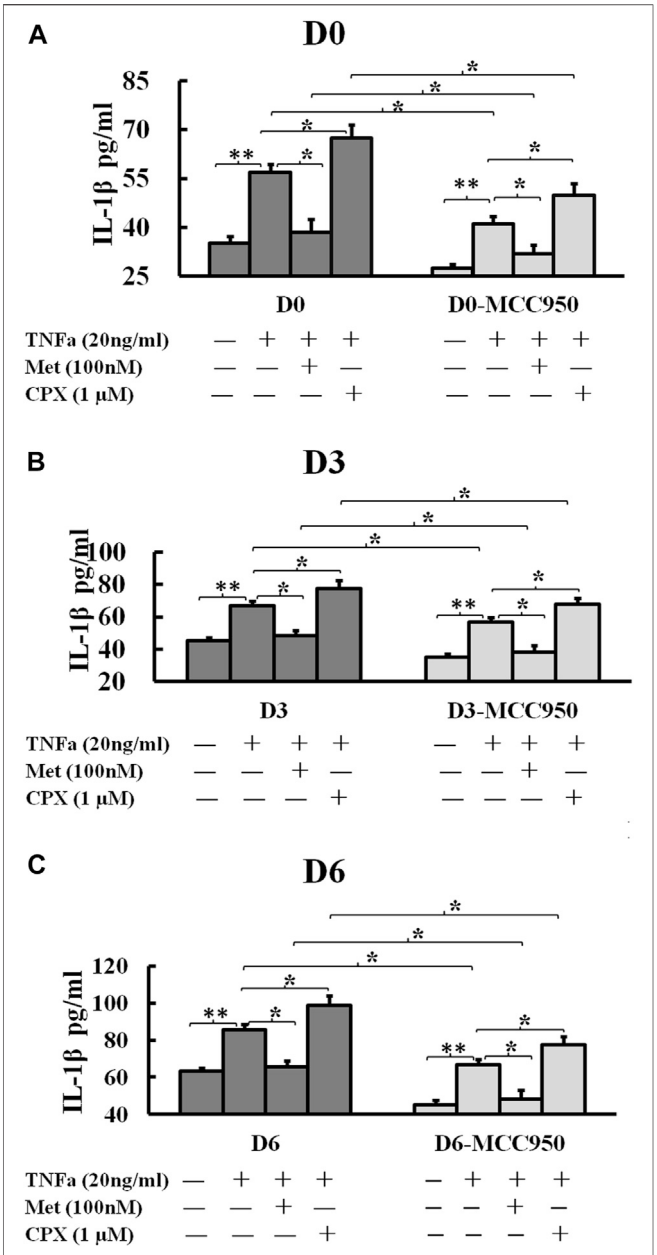

FIGURE 6 | Histamine H_3 receptor modulates the secretion of IL-1β. C2C12 cells were incubated in the presence of TNFa (20 ng/ml), TNFa + Met (100 nM) or TNFa + CPX (1 μM) or MCC950 (1 μM) for 24 h at D0, D3, or D6. The mature IL-1β protein secretions were assessed by ELISA. $^{*}p < 0.05$, and $^{**}p < 0.01$ between two compared groups. **(A)**: IL-1β secretion in D0 cells. **(B)** IL-1β secretion in D3 cells. **(C)** IL-1β secretion in D6 cells. Ctrl, control; TNFα, murine Tumor Necrosis Factor-alpha; Met, methimepip; CPX, ciproxifan; IL-1β, interleukin 1beta.

DISCUSSION

Inflammation is an immunological response to injury and the concomitant release of damage associated molecular patterns (Hofer et al., 2014). The underlying molecular mechanisms are complex but recent studies implicate activation of inflammasomes in sterile inflammatory reactions (Mangan et al., 2018). The present study verified the presence of functional NLRP3-inflammasome in mouse striated C2C12 myocytes (Cho and Kang, 2015; Gallo et al., 2015). TNFα induced both the expressions of pro-IL-1β and also the activation of NLRP3 inflammasome in C2C12 myocytes resulting in secretion of the mature IL-1β protein. TNFα also reduced cell viability and the expression of myogenesis markers, suggesting that it inhibited myocyte differentiation. These findings are in line with previous studies which have shown that IL-1β induces markers of atrophy in C2C12 cells (Huang et al., 2017). Together these findings suggest that TNFα mediated inflammasome activation with ensuing IL-1β secretion could play an important role in postinjury muscle inflammation and inflammation associated muscle atrophy.

Stimulation of H_3R signaling by Met attenuated the TNFα-induced activation of NLRP3 inflammasome, whereas, inhibition of H_3R signaling enhanced the inflammasome activation by TNFα. Recently Gao et al. showed that loratadine, a histamine H_1 receptor blocker, inhibits the activation of NLRP3 inflammasome (Gao and Zhang, 2020), but there are no studies addressing the role of H_3R in regulation of NLRP3 inflammasome. The effect of TNFα on myogenic differentiation is affected by the conditions used and also on concentration of TNFα. Low levels of TNFα are required for the differentiation, but on the other hand, especially at higher concentration, such as during inflammation, TNFα has been shown to increase myoblast proliferation, and to prevent the cells entering to cell cycle arrest that is a prerequisite for the initiation of myoblast differentiation (Grounds and Torrisi, 2004; Chen et al., 2007; Alter et al., 2008). TNFα reduced the expression of the earliest differentiation markers on the earlier day three time point slightly more than at later sixth day time point. The differences between the time points are probably explained by the number of the cells that have entered the cell cycle arrest before TNFα treatment, because TNFα does not inhibit the differentiation after the cells have entered the cell cycle arrest (Alter et al., 2008), and by day sixmore cells had entered the cell cycle arrest and started differentiation than by day three, and probably therefore the effect of TNFα on differentiation was slightly mitigated.

Activation of H_3R further slowed myogenic differentiation in the presence of TNFα. The mechanism remains to be elucidated but one possibility is that H_3R activation directs cell resources into processes of reducing inflammation, thus halting the differentiation. In carcinoma cells H_3R -mediated activation of protein kinase C α inhibited the growth of cholangiocarcinoma and hepatocellular carcinoma cells (Francis et al., 2009; Yu et al., 2019). The finding that blocking the H_3R by CPX in the presence of TNFα enhanced cell differentiation, is also in line with studies on cancer cells reporting that inhibition of histamine receptor H_3 suppresses the growth and metastasis in human non-small cell lung cancer cells (Zhao et al., 2020) thus potentially promoting differentiation. Moreover, the enhanced inflammation induced by CPX + TNFα could also direct cells into apoptosis resulting in the release of ATP, which further activates the NLRP3 inflammasome (Jo et al., 2016).

CONCLUSION

TNFα induces NLRP3 inflammasome activation and inhibits myogenesis in striated C2C12 cells during myogenic

differentiation. Histamine H_3R signaling slowed the differentiation and inhibited TNFα induced NLRP3 inflammasome activation. Inhibition of H_3R signaling enhanced the differentiation and TNFα-induced inflammation. These findings implicate a regulatory role for histamine H_3R in NLRP3 inflammasome activation. This beneficial effect of H_3R activation should be balanced with the potentially disadvantageous effect of further slowing down the myocyte differentiation in the presence of TNFα. H_3R modulation could represent a lucrative target in the treatment of postinjury muscle inflammation and atrophy.

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 author.

AUTHOR CONTRIBUTIONS

All authors substantially contributed to the drafting and revising of the article, as well as the final approval of the version to be submitted. YC got the financial supports from the Foundations, conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures, reviewed drafts of the paper, redid the experiments and revised the paper. YM repeated the experiments, analyzed the data, wrote the paper, prepared figures, and reviewed drafts of the paper. JF performed the experiments, analyzed the data, wrote the paper and reviewed drafts of the paper. YW performed the experiments, and reviewed drafts of the paper. TL contributed reagents, materials and analysis tools, and reviewed drafts of the paper. KN contributed reagents, materials and analysis tools, and reviewed drafts of the paper. KE helped to design the experiments, and reviewed drafts of the paper. JW provided the experimental platform and all the equipments, conceived and designed the experiments, and reviewed drafts of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.599393/full#supplementary-material

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frontiers in Pharmacology

BRIEF RESEARCH REPORT
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Effect of Intravenous IgM-Enriched Immunoglobulins on Presepsin and Other Sepsis Biomarkers

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Patients in septic shock with low IgG and IgM serum concentrations have higher mortality rates compared to those with normal immunoglobulin levels and, therefore, there is a rational explanation to administer intravenous IgM-enriched immunoglobulins to septic patients in ICU. Aim of this study is to evaluate the effectiveness of intravenous IgM-enriched immunoglobulins in decreasing several sepsis biomarker concentrations. 26 sepsis patients were enrolled in this observational cohort study and Nitric Oxide, Endocan, Pentraxin and presepsin serum levels were measured during their first 3 days of ICU stay. The use of intravenous IgM-enriched immunoglobulins did not influence the temporal evolution of SOFA, Nitric Oxide, Endocan, Pentraxin and Presepsin in the first 3 days of ICU stay in a statistically significant manner, even if Presepsin decreased of 25% from day 1 to day 2 in the Pentaglobin group. It seems possible that Pentaglobin infusion reduces the Presepsin level in a more effective way if it were administered to a younger population (p = 0.012). In conclusion, age modifies the response of Presepsin to Pentaglobin and is a critical variable when investigating the effect of intravenous IgM-enriched immunoglobulins on sepsis.

Keywords: presepsin (soluble CD14-subtype), sepsis - diagnostics, septic shock (MeSH), immunoglobulin M, elderly

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INTRODUCTION

Elderly patients are predisposed to an increased rate of sepsis because of their state of immunosenescence and they suffer a more prolonged proinflammatory response compared to younger patients. Despite seniors have the capability to preserve antibodies against previously exposed antigens, they have a decreased ability to produce specific opsonophagocytic antibodies against neoantigens (new antigens) (Nasa et al., 2012).

Patients in septic shock with low IgG and IgM serum concentrations have higher mortality rates compared to those with normal immunoglobulin levels (Myrianthefs et al., 2010).

Many preparations, including intravenous IgM-enriched immunoglobulins, are available with a growing number of accepted uses, since these products are generally well-tolerated and with few side effects (Biagioni et al., 2021).

Polyclonal intravenous immunoglobulins have pleiotropic effects on inflammatory and immune mechanisms and have been proposed as adjuvant therapy to modulate inflammatory processes. During the SARS epidemic in Hong Kong, e.g., in selected cases, Tsui et al. (2003), and Ho et al. (2004) administered intravenous IgM-enriched immunoglobulins (Pentaglobin). One case of

COVID-19 treated with polyclonal preparation of IgM as adjuvant therapy (Carannante et al., 2020) has been published.

Nevertheless, in 2016, Surviving Sepsis Campaign guidelines suggested against immunoglobulins use in sepsis because the available evidence was not clearly sufficient to support their widespread use in the treatment of sepsis (Rhodes et al., 2017). However, there is new experimental evidence (Vaschetto et al., 2017) supporting the rationale for IgM enriched immunoglobulin solution use in sepsis patients, and at the same time, no specific drug or strategy up to now has proven to be efficacious in reducing all causes sepsis mortality. Results from recent trials not included in the Cochrane metanalysis used by the SSC expert in the 2016, indicate that intravenous IgM-enriched immunoglobulins may be effective in septic patients (Cavazzuti et al., 2014; Giamarellos-Bourboulis et al., 2016). Cui et al. (2019) in a meta-analysis published in 2019, including 19 studies (1,530 patients), estimated that mortality rates were significantly lower in patients who received intravenous IgM-enriched immunoglobulins than in their respective control groups [relative risk (RR) 0.60; 95% confidence interval (CI) 0.52–0.69]. An ongoing trial (Biagioni et al., 2021) aims to discriminate if a personalized dose based on patients serum IgM versus standard dose is more efficacious in the treatment of septic shock (IgM-fat trial registered on ClinicalTrials.gov (NCT04182737) on December 2, 2019).

On the other hand, the search for reliable and precocious biomarkers of sepsis is never ending: procalcitonin and presepsin are widely used to diagnose sepsis and guide antimicrobial therapy, but the list could include also other molecules which may be relevant to understand the physiopathology of sepsis, as Nitric Oxide (NO), Endocan, Pentraxin (Supplementary material file). We aimed to prove the efficacy of intravenous IgM-enriched immunoglobulins by using indirect indicators of outcome; our hypothesis is that treated patients should benefit of a lower level of sepsis biomarkers (presepsin, endocan, pentraxin, plasmatic Nitric Oxide), compared to not treated subjects.

METHODS

The protocol for this study was reviewed and approved by the Ethics Committee Campania Sud as no profit research (protocol N. 165/2018).

Twenty-six consecutive patients with sepsis and septic shock were admitted to the University of Salerno ICU and enrolled in this observational cohort study. Based on the decision of the physician in charge, patients received an intravenous immunoglobulin preparation (Pentaglobin) in addition to standard therapy, or received standard therapy only (fluids, vasopressors, antibiotic therapy, parenteral and/or enteral nutrition) and were considered as the control group. The treatment with Pentaglobin for patients with septic shock was chosen always according to the physicians judgment, with exclusion of those presenting hypersensitivity to the IgM enriched preparation in use or its excipients, or had previous intravenous immunoglobulin therapy, selective absolute IgA deficiency with antibodies to IgA, pregnancy or breastfeeding or a positive pregnancy test. We did not include patients for whom clinical decision to withhold life-sustaining treatment was taken or when the diagnosis of shock was uncertain. Pentaglobin therapy started on the same day sepsis was diagnosed; 5 ml/kg Pentaglobin per day was infused for 72 h. Left over blood samples for NO, Pentraxin and Endocan were acquired daily for 3 days, the first sample was collected before the IgM enriched solution infusion and the third was collected after its end. After centrifugation at room temperature using 2500 G-force for 10–15 min, serum was transferred in 1–2 ml aliquots tubes, which were stored at −80°C until assayed by using a commercial solid-phase enzyme-linked immunosorbent assay (ELISA).

During the 1-year study period, presepsin was part of the routine clinical monitoring, measured by a commercial instrumentation on whole blood (PATHFAST Presepsin chemiluminescent enzyme immunoassay). Severity of critical illness and development of organ failure were assessed daily by sequential organ failure assessment (SOFA) score.

Statistical Analysis

Data were summarized as mean and standard deviation or as median and interquartile range and the between-groups comparisons were performed by independent T-Test or Mann-Whitney Test, as appropriate. The between-groups differences (Pentaglobin treated patients versus controls) of the biomarkers evolution over time were investigated by linear mixed models (LMM) analysis. The effect modification by age on the difference in presepsin levels between Pentaglobin treated and untreated patients was investigated by the effect modification analysis, i.e. by introducing into the same multiple LMM (with presepsin as dependent variable), age (in years), treatment (1 = pentaglobin; 0 = no pentaglobin) and their interaction term (age x treatment). Given the fact that methodologists recommend to include into a multiple regression model 1 variable every approximately 5-9 patients (Vittinghoff and McCulloch, 2007), the optimal sample size requested for including the three variables mentioned above (that is, age, treatment, and age x treatment) was 26 patients. Such a sample size allows to adjust for 3 variables, i.e., 1 variable every approximately 9 enrolled patients. In the multiple LMM, age was considered either as confounder or as an effect modifier on the basis of clinical consideration. The difference in presepsin levels between Pentaglobin treated and untreated patients at pre-defined values of age was calculated by the linear combination method which allows to calculate the effect of Pentaglobin by assuming all the patients as having a specific age (40, 50, 60, 70, and 80 years). Data analysis was performed by SPSS for Windows version 22, IBM, Chicago, Illinois, United States.

RESULTS

15 septic shock patients were treated with Pentaglobin (males 40%) and 11 patients were treated with standard therapy (males: 45%). The characteristics of the study population are summarized in **Table 1**. The decision to treat with Pentaglobin was left to the physician in charge, independently from the purpose of this research. Included patients had a median age of 66 years (interquartile range 60–81); median SOFA score at ICU admission was 7 (interquartile range 5–11); median SAPS was 25 (interquartile range 17–35). Physicians decided to administer

TABLE 1 | Patients' characteristics and Predisposition, Infection/injury type, Response and Organ dysfunction (PIRO) score. The PIRO score includes eight variables, each given the same weight (Age, Comorbid conditions, Leukopenia, Hypothermia, Cardiovascular dysfunction, Respiratory dysfunction, Renal dysfunction, Central nervous system dysfunction).

	n. patients	Age (media)	Male	Female	Dead/alive	Predisposition	Infection (lung/abdomen)	SAPS II
Controls	11	71 ± 16	5	6	9/2	4	2/9	2728
Pentaglobin group	15	65 ± 16	6	9	9/6	10	3/12	2946

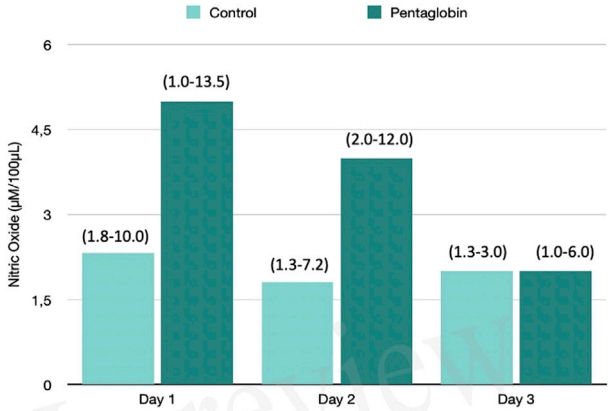

FIGURE 1 | Comparison of Nitric Oxide changes over time between Pentaglobin and control groups by linear mixed models ($p = 0.83$). The height of bars represents the median values and the numbers in brackets the corresponding interquartile range of Nitric Oxide (NO) levels determined by ozone-chemiluminescence technology in blood samples in patients receiving intravenous immunoglobulin preparation (Pentaglobin) and in those of the control group. NO was collected for the first 3 days from sepsis diagnosis after ICU admission. The use of Pentaglobin did not significantly influence the temporal evolution of NO ($p = 0.83$) versus the standard therapy, although a decreasing trend of NO level at the end of the third day by more than 50% was observed in the treated group compared to the control group. The between-groups comparison over time was performed by linear mixed models. See text for more details.

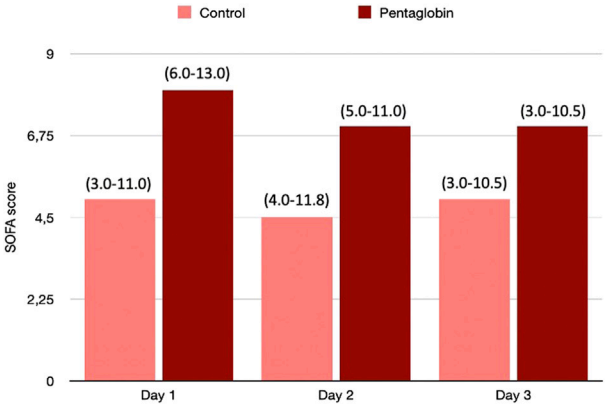

FIGURE 2 | Comparison of SOFA changes over time between Pentaglobin and control groups by linear mixed models ($p = 0.49$). The height of bars represents the median values and the numbers in brackets the corresponding interquartile range of SOFA scores calculated daily to assess the severity of critical illness and the development of organ failure in Pentaglobin treated patients during the first 3 days of treatment after sepsis diagnosis compared with the control group. There was no significant difference in the SOFA score at admission between the Pentaglobin group and controls ($p = 0.09$). Furthermore, the use of Pentaglobin did not influence the temporal evolution of SOFA ($p = 0.49$) versus the standard therapy. See text for more details.

Pentaglobin to a more severe subset of patients: mean (and SD) SOFA score at admission was 18.6 ± 4.5 in Pentaglobin treated patients versus 9.5 ± 4.0 of the control group, but this difference was not statistically significant ($p = 0.09$). There was no substantial age difference between Pentaglobin and control group [mean age was 65 ± 16 (SD) years in Pentaglobin group and 71 ± 16 (SD) years in the control group; $p = 0.35$].

Effect of Pentaglobin on the Temporal Evolution of SOFA, NO, Endocan and Presepsin

The use of Pentaglobin did not influence the temporal evolution of SOFA ($p = 0.49$), NO ($p = 0.83$), Endocan ($p = 0.38$), PTX ($p = 0.96$) and presepsin ($p = 0.20$) in the first 3 days of ICU stay (**Figures 1–4**). However, Presepsin, decreased by 25% from day 1 to day 2 in the Pentaglobin group, whilst the decrease was slightly lower in the control group (22%). The between-groups difference was not statistically significant (**Figure 5**).

Effect Modification by Age on the Relationship Between Pentaglobin and Presepsin

Nevertheless, by applying linear mixed models, we calculated the Pentaglobin effect by assuming that all the patients had the same age, to overcome the limited number of patients in each decade. If all patients had had 40 years old, the difference in presepsin concentration between pentaglobin treated and control patients would have been higher rather than in patients of 50, 60, 70 or 80 years of age. In other words, age significantly modified ($p = 0.012$) the presepsin response to pentaglobin (**Figure 6**), the difference in presepsin levels between Pentaglobin treated and untreated patients being closely dependent on age. In fact, the higher the age, the lower the difference in presepsin levels between Pentaglobin treated and untreated patients.

DISCUSSION

The incidence of sepsis and septic shock is increasing in the elderly. They are predisposed to sepsis, due to the effects of aging

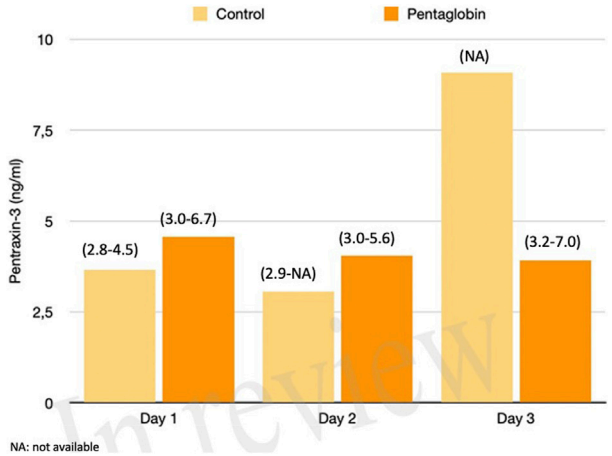

FIGURE 3 | Comparison of Pentraxin-3 changes over time between Pentaglobin and control groups by linear mixed models ($p = 0.96$). The height of bars represents the median values and the numbers in brackets the corresponding interquartile range of Pentraxin-3 levels between Pentaglobin treated patients and controls during the first 3 days of ICU stay from sepsis diagnosis. PXT-3 levels increased daily up to the third day in septic patients treated with standard therapy (i.e., the control group) in agreement with the role of this acute-phase protein in the early phase of sepsis. The between-groups comparison over time was performed by linear mixed models and resulted to be not significant ($p = 0.96$). See text for more details.

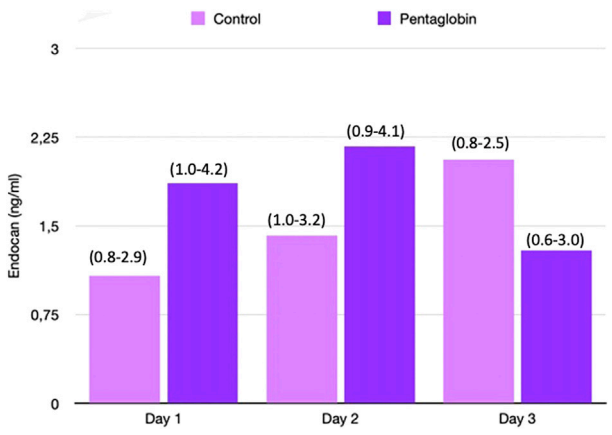

FIGURE 4 | Comparison of Endocan changes over time between Pentaglobin and control groups by linear mixed models ($p = 0.38$). The height of bars represents the median values and the numbers in brackets the corresponding interquartile range of Endocan in Pentaglobin treated patients and in controls in the first 3 days from ICU admission and septic shock. Pentaglobin therapy seems to restrain a regular upward trend of the Endocan levels, which instead characterizes the first 3 days of septic patients who were only on conventional therapy. The between-groups comparison over time was performed by linear mixed models and resulted to be not significant ($p = 0.38$). See text for more details.

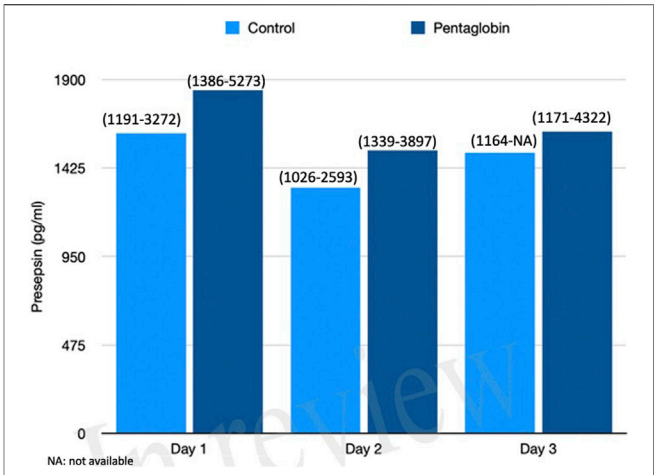

FIGURE 5 | Comparison of Presepsin changes over time between Pentaglobin and control groups by linear mixed models ($p = 0.20$). The height of bars represents the median values and the numbers in brackets the corresponding interquartile range in Pentaglobin treated patients and in controls. Presepsin was measured for the first 3 days from diagnosis of septic shock and the figure shows decrease in its concentration of 25% from day 1 to day 2 in the Pentaglobin group, whilst the decrease was slightly lower in the control group. The between-groups comparison over time was performed by linear mixed models and resulted to be not significant ($p = 0.20$). See text for more details.

itself and to the sum of co-morbidities, prolonged hospitalizations, functional limitations. Management is largely based on standard international guidelines while there is evidence that several drugs, i. e., antibiotics, need special care, since the pharmacokinetic parameters changes with aging and the side effects can differ. In the elderly, serum IgM concentrations are significantly reduced, whereas serum IgA concentrations are maintained (Lock and Unsworth, 2003). Nevertheless, no significative effect of Pentaglobin on sepsis biomarkers in elderly patients was detected, while statistical analysis addressed the greater potential benefit in a younger group. In literature, adverse effects from intravenous immunoglobulins have been reported and can be classified into three types according to their onset: immediate, delayed, and late onset. Immediate adverse effects occur during infusion, for example anaphylactoid reactions; delayed adverse effects occur hours or days after infusion, for example pulmonary, renal, haematologic and neurologic events; and late adverse effects include transmission of infectious agents such as hepatitis C and prion diseases (Nydegger and Sturzenegger, 1999). The present research did not report any adverse event related to the use of Pentaglobin. The surviving sepsis campaign (SSC) guidelines (Rhodes et al., 2017) suggest early eradication of septic foci, administration of anti-infective agents, maintenance of hemodynamic stability through fluid administration and vasopressors. The SSC guidelines remain the cornerstone of treatment for sepsis and the fact that in 2016, SSC guidelines suggested against Ig use in sepsis, because of weak evidence of efficacy from previous studies, surely reduced the number of treated patients in our ICU, discouraging physicians to prescribe a drug which is safe but not surely helpful. However, results from successive trials (Giamarellos-Bourboulis et al., 2016) and systematic meta-analyses (Cui et al., 2019) indicate that intravenous IgM-enriched

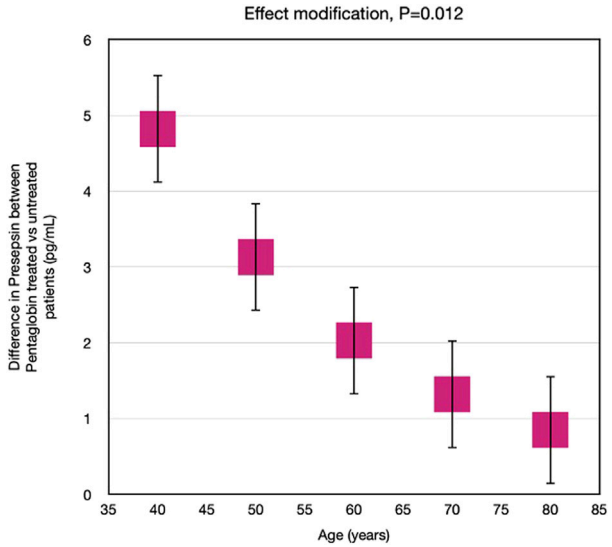

FIGURE 6 | Effect modification by age on the effect of Pentaglobin on presepsin. The effect of Pentaglobin for increasing Presepsin, compared to the control group, was strictly dependent on age. Through an advanced statistical technique (linear mixed models), the effect of Pentaglobin assuming that all the patients in the study were of a certain age was estimated (such an approach allows to overcome the problem of the reduced sample size in the various age groups). If all patients were 40 years old, the difference in presepsin (Pentaglobin treated patients versus controls) would be 4.82, if they were 50 years old the difference in presepsin would be 3.13, if they were 60 years old the difference in presepsin would be 2.03, if they were 70 years old the difference in presepsin would be 1.32, if they were 80 years old the difference in presepsin would be 0.86. Therefore, the reduction of presepsin determined by Pentaglobin is greater the lower is the age. The difference in the effect of the drug between the various ages is statistically significant (p = 0.012). Data are mean differences and 95% CI.

immunoglobulins reduced mortality (Cui et al., 2019) and the addition of IgM enriched solution to the SSC bundles appears as a promising treatment option, in particular in those patients with an acute disease onset, who are heavily inflamed, showing signs of overt septic shock and those with an immunocompromised phenotype, (patients in an immunosuppressive stage) (Nydegger and Sturzenegger, 1999; Nierhaus et al., 2020).

The main limitation of this study is the low sample size, even if we systematically collected all the sepsis shock patients admitted to our ICU during the study period. This pilot study needs a follow up, with a focused clinical trial, aimed to understand the role of age in IgM infusion efficacy.

CONCLUSION

In this limited case series, we found no evidence that Pentaglobin has a direct and significant effect on the investigated sepsis biomarker levels. Nevertheless, age modifies the response of Presepsin to Pentaglobin and can be considered as a critical variable whilst investigating the effect of intravenous IgM-enriched immunoglobulins on sepsis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Campania SUD. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

OP: conceptualization; GS and DB data collection, MB experimental laboratory procedures, GT statistics. All authors contributed to manuscript revision, read, 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/fphar.2021.717349/full#supplementary-material

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Latest Progresses in Allergic Diseases Biomarkers: Asthma and Atopic Dermatitis

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In the last years, the understanding of the pathologic mechanisms of asthma and atopic dermatitis, both characterized by allergic inflammation, has greatly improved. However, it is evident that both diseases present with high heterogeneity, which complicates the diagnosis and the therapeutic approach of the patients. Moreover, some of the currently available strategies to treat asthma and atopic dermatitis are still mostly controlling the symptoms, but not to lead towards full healing, thus having these two diseases labelled as unmet clinical needs by WHO. Therefore, the "one-size-fits-all" strategy is outdated for asthma and atopic dermatitis, and there is the need of better methods to clearly diagnose the disease and tailor the therapy according to the specific symptomatology. In this regard, the use of biomarkers has been advanced in order to characterize both diseases according to their clinical signs and to facilitate the subsequent treatment. Despite the advancements made in this regard, there is still need for better and more sensitive biomarkers and for less invasive sampling methodologies, with the aim to diagnose specifically each manifestation of asthma and atopic dermatitis and to provide the best treatment with the least suffering for the patients.

Keywords: allergic inflammation, asthma, atopic dermatitis, biomarkers, eosinophils, neutrophils

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INTRODUCTION

In recent years, advancements of medical research in the field of allergic diseases have led to a better understanding that pathologies characterized by allergic inflammation (AI) are heterogeneous and present with a high degree of variability among patients (Roth and Stolz, 2019; Bakker et al., 2020). Moreover, most of the currently available treatments are still only able to ease the symptomatology/symptoms, to decrease inflammation and some of them to partially prevent exacerbations and perhaps to modify the natural course of the disease. However, even the newest biologic-based drugs are not able to cure it. This might be due to the concomitant presence of an atopic condition together with the inability of the atopic individual to fully resolve the inflammation. Thus, allergic diseases and especially severe asthma and atopic dermatitis have been labelled by the WHO as unmet clinical needs (Breiteneder et al., 2019). Given the advances in research, and in light of the concept of personalized medicine, the necessity of finding novel and more accurate biomarkers for allergic diseases has been raised. A biomarker (or biological marker) is defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" according to the National Institute of Health. Consequently, biomarkers evaluation should help in the diagnosis of the disease as well as in predicting its outcomes and the effects of the prescribed therapy (Narendra et al., 2019).

BIOMARKERS FOR ASTHMA

Asthma is a lung disease characterized by sometimes irreversible bronchoconstriction, airway hyperresponsiveness, chronic inflammation, mucus hypersecretion and tissue remodeling (Lambrecht and Hammad, 2015). In the last 40 years its prevalence and morbidity have increased, with approximately 300 million individuals affected worldwide and a total of $80 billion dollars yearly expenses (Narendra et al., 2019). Over the years, several attempts have been made to better characterize the etiopathology of the disease, but still there is no effective therapy for all the spectrum of asthma forms, especially for the severe ones. Indeed in mild/moderate cases, asthma symptoms and underlying inflammation can mostly be controlled with the use of inhaled β-adrenergic agonists, muscarinic antagonists and glucocorticosteroids and other available anti-inflammatory drugs (Lambrecht and Hammad, 2015; Koarai and Ichinose, 2018). More recently, the use of monoclonal antibodies such as anti-IgE, anti-IL-5, anti-IL-5R, and anti-IL-4/IL-13Rα has been demonstrated to be able to control asthma pathogenesis and hence symptoms, but not to resolve the disease. Thus, as mentioned above, asthma remains an unmet clinical need.

Asthma Endotypes and Phenotypes

In recent years, asthma has been defined as a disease characterized by heterogeneous features, which include the type of inflammation, presentation of the symptoms, response to treatments and long-term consequences for the patients. Therefore, asthma is better characterized by defining endotypes and phenotypes (Narendra et al., 2019; Roth and Stolz, 2019). Asthma endotypes encompass the pathologic mechanisms underlying the disease, while phenotypes include its clinical manifestations (Kuruvilla et al., 2019). Endotypes classification allows asthma to be divided into type 2 and non-type 2 asthma, although there is evidence of different subtypes linked to the inflammasome and skin structural components, and mixed 2, 1, 17 subtypes (Agache and Akdis, 2019). Asthma phenotypes include patients' features (age, gender, ethnicity, etc.), morphophysiological characteristics of the airways, response to therapies and clinical outcomes (Agache and Akdis, 2019). The emergence of the concept of asthma endotypes and phenotypes has prompted the need of a better understanding of the disease characteristics, in order to have a more personalized therapeutic approach and to predict the outcomes of the treatment. Therefore, the use of biomarkers to define the specific presentations of asthma has been advanced, each of them with their pros and cons.

Biomarkers for Asthma

In order to be eligible for asthma, a biomarker should be "superior, actionable, valuable, economical, and clinically deployable" (Diamant et al., 2019). Biomarkers for asthma are mainly divided into biomarkers for type 2 and non-type 2 asthma and might be sampled from different sources, with several advantages and disadvantages (Diamant et al., 2019; Narendra et al., 2019).

Biomarkers for Type 2 Asthma

Eosinophils

One of the main biomarkers for type 2 asthma is the eosinophil (Eos) numbers, which are preferably analyzed in the blood and in the sputum of asthmatic patients due to the lower invasiveness of these methods, although with lower reproducibility and higher technical complexity (Diamant et al., 2019). Increased Eos counts in the blood (>400 cells/μl) were associated with higher prevalence of exacerbations and lower possibilities to control the disease (D. B. Price et al., 2015). However, Eos blood counts for asthma are not fully reliable, since blood eosinophilia might be due to other T2 inflammation-inducing conditions, such as parasitic infections or some autoimmune diseases (Narendra et al., 2019). Nevertheless, blood eosinophilia is a good marker to follow the T2 inflammation after treatment with anti-IL-5 (mepolizumab and reslizumab), anti-IL-5Rα (benralizumab) and anti-IL-4 (dupilumab) biologics (Castro et al., 2011; Wenzel et al., 2013; Ortega et al., 2014; FitzGerald et al., 2016), as high blood Eos count is considered a good predictive value for the response to the aforementioned biologics (Cevhertas et al., 2020). Sputum Eos is believed to be the most accurate method to assess eosinophilic asthma (a value higher than 2% Eos is considered indicative of airway inflammation) (Westerhof et al., 2015; Walsh et al., 2016). A more refined technique to distinguish between type 2 and non-type 2 asthma involves analysis of the sputum mRNA levels of Th2 cytokines (Seys et al., 2017). Sputum Eos have been historically employed to follow the outcomes of corticosteroid treatment, since lower eosinophilia correlated with reduced exacerbations and hospitalizations after inhaled corticosteroids (ICS) administration (Morrow Brown, 1958; Green et al., 2002; Fitzpatrick et al., 2016). Another technique employed to analyze Eos in asthmatic patients is bronchoscopy, which is performed via biopsies, bronchoalveolar lavage or bronchial brushing. However, the higher invasiveness and complexity of these techniques limits their application (Diamant et al., 2019). Another possibility involves measurement of Eos granule proteins, such as Eos peroxidase, Eos cationic protein and Eos-derived neurotoxins, which have been found to decrease after administration of anti-Eos biologics (Narendra et al., 2019).

Fraction of Exhaled Nitric Oxide

FeNO measurement is an indirect indication of airway inflammation, since it correlates with Eos counts in the lungs (Fajt and Wenzel, 2015). Indeed, FeNO is linked to eosinophilia, according to the American Thoracic Society recommendations, (>50 ppb and >30 ppb for adults and children, respectively) (Dweik et al., 2011). FeNO is directly measurable in the exhaled breath of patients. The sampling method is easy and non-invasive, and the results are reproducible, prompting its use also for pediatric asthma (Neerincx et al., 2017). However, the results might be influenced by several factors, such as age, smoking habits, drug use, which should be taken into consideration when performing the measurement (Buchvald et al., 2005; Borrill et al., 2006). Even though it was found that FeNO levels decrease in response to inhaled corticosteroids (ICS)

and dupilumab treatment (D. Price et al., 2013; Wenzel et al., 2013), the ERS/ATS guidelines suggest to avoid the use of FeNO as a predictive marker for therapy in severe asthma (Chung et al., 2014).

Serum IgE

Total serum IgE were found to be increased in allergic asthmatic adults and children, and their levels increased with disease severity (Burrows et al., 1995). Moreover, high serum IgE levels are indicative of sensitization to an allergen, which makes patients eligible for treatment with the monoclonal anti-IgE antibody omalizumab. However, it was demonstrated that, despite reducing serum IgE, total IgE levels do not change significantly after omalizumab administration, due to the fact that omalizumab binds to free IgE and forms complexes with them, thus increasing the total IgE levels (Humbert et al., 2014). However, its use was found to reduce the incidence of asthma exacerbations (Humbert et al., 2014). Therefore, this marker does not allow to predict the response to treatments in asthmatic patients, and must be analyzed together with other biomarkers.

Periostin

Periostin is produced and released by epithelial cells after stimulation with IL-13, a cytokine indicative of Th2 inflammation (Izuhara et al., 2016). Periostin's effectiveness as a biomarker was shown to be higher than Eos, FeNO and serum IgE in patients with uncontrolled severe asthma and ICS treatment (Jia et al., 2012). Moreover, patients with high periostin levels were found to show reduced asthma exacerbations after treatment with lebrikizumab (anti-IL-13), demonstrating the prognostic value of serum periostin concentrations (Hanania et al., 2015). However, its levels fluctuate with age (Narendra et al., 2019) and can change also during other inflammatory processes such as atopic dermatitis, eosinophilic esophagitis and cancer, requiring its use in addition to other markers (Izuhara et al., 2016). In addition, the existence of different splicing variants of periostin complicates the measurement of this biomarker.

Biomarkers for Non-type 2 Asthma

Neutrophils

Higher neutrophil counts in the sputum and in the blood have been associated with severe forms of asthma (Moore et al., 2014; Ricciardolo et al., 2018). However, there is no clear definition of neutrophilic asthma, since different threshold values for neutrophil levels were reported in the literature (Simpson et al., 2006; Moore et al., 2014). Moreover, airway neutrophilia was also found to be induced by use of oral corticosteroids (Alam et al., 2017), but also by other conditions, such as obesity, smoking habits, gastroesophageal reflux or lung infections (Ray and Kolls, 2017). This complicates the diagnosis of neutrophilic asthma, since some of its features are mistakenly ascribed to other diseases, such as chronic obstructive pulmonary disease, or effects of smoking (Gibson and Foster, 2019). Generally, a value between 61 and 76% is considered indicative of neutrophilic airway inflammation, although no real consensus exists about these threshold values (F. Schleich et al., 2016).

Serum Cytokines

The main markers associated with neutrophilic asthma are IL-17, IL-8, and TNFα, since their levels in the serum were found increased in asthmatic patients with neutrophilia (Diamant et al., 2019). A positive correlation was found between neutrophil numbers and IL-17 mRNA levels in the sputum (Bullens et al., 2006), and it was found that bronchial epithelial cells released IL-8 after stimulation with IL-17, contributing indirectly to IL-8-induced neutrophils recruitment (Lindén, 2001). However, this marker did not show any therapeutical predictive value since treatment with an anti-IL-17R biologic, brodalumab, did not prove to be effective in severe asthma (Busse et al., 2013). Tightly linked to airway neutrophilia is the cytokine IL-8, since it is known to be a chemoattractant for neutrophils and it has been found in high levels in BAL and sputum uncontrolled asthmatic patients (Gao et al., 2017). Moreover, expression of IL-8 receptors CXCR1-2 was found to be increased in the sputum of neutrophilic asthmatic patients (Wood et al., 2012). In this regard, the use of CXCR2 antagonists was shown to decrease neutrophils count in the sputum and to reduce mild exacerbations (Nair et al., 2012), further bolstering the connection between neutrophils and asthma. Other cytokines involved in neutrophilic asthma include IL-4, which might indirectly induce neutrophil migration and activation by regulating the expression levels of IL-8, TNF-α, and IL-1β in correlation with the severity of the disease (Lavoie-Lamoureux et al., 2010). Therefore, IL-8 and IL-4 might be helpful in distinguishing between airway neutrophilia due to asthma or other pathological conditions. TNFα levels were found to be increased in the sputum of neutrophilic asthmatics (Simpson et al., 2007) and to positively correlate with both NO and neutrophil numbers in severe asthmatic patients (Silvestri et al., 2006). Interestingly, treatment with the anti-TNFα etanercept improves airway hyperresponsiveness and quality of life in refractory asthma, and this improvement positively correlated with etanercept-induced reduction of membrane-bound TNFα expression (Brightling et al., 2008). Thus, TNFα might be used also as a predictive biomarker for etanercept therapy. Other markers are related to neutrophil activation and include sputum myeloperoxidase and elastase, which were detected in high levels in neutrophilic asthma (F. Schleich et al., 2016).

Novel Biomarkers

Airway Remodeling

One of the main features of asthma is the airway remodeling, involving airways obstruction, mucus hypersecretion, angiogenesis, and excessive fibrosis (Bergeron et al., 2009). Unfortunately, at the moment there is no clear-cut marker for airway remodeling, and the preferred method to analyze remodeling in the airways is the bronchial biopsy, which is highly invasive and risky. Furthermore, the great variability in the tissue raises the necessity of having more than one sample (Diamant et al., 2019). Some less-invasive markers include

sputum matrix metalloproteinase 2 (MMP-2), fibroblast growth factor 2 (FGF-2) and galectin-3, the latter being predictive of omalizumab effects on airway remodeling (Mauri et al., 2014; Elkolaly and Ali, 2018; Sivakoti et al., 2018; Tan et al., 2020). Other airways remodeling markers include CCL16, released by bronchiolar exocrine cells, which is usually measured in the sputum and compared to IL-8 levels (F. Schleich et al., 2016).

Volatile Organic Compounds

VOCs are a collection of molecules derived from the metabolism of different endogenous or exogenous compounds. These molecules were found to differ between eosinophilic and neutrophilic asthma. For example, hexane and 2-hexanone were found to be characteristic of eosinophilic asthma, with similar accuracy to FeNO and blood Eos (F. N. Schleich et al., 2019). On the other hand, the molecules found in high concentrations in neutrophilic asthma are nonanal, 1-propanol and hexane (F. N. Schleich et al., 2019).

Specialized Pro-resolving Mediators

SPMs are a class of lipid molecules encompassing different families with their biosynthetic pathways and receptors, all of them implicated in the resolution of inflammation (Fullerton and Gilroy, 2016). SPMs levels might be analyzed in a wide number of biological materials, such as blood, sputum, bronchoalveolar lavage (BAL), exhaled breath condensates, as well as in urine, breast milk and tears, in their bioactive concentration (pg/ml) (Serhan, 2014). Notably, SPMs pathways are reduced in severe asthma patients. Specifically, lower lipoxin A4 (LXA4) levels were found in the BAL of severe asthmatic patients (Planaguma et al., 2008), correlating with decreased lung functions. Moreover, severe asthma patients have been found to present with reduced docosahexaenoic acid (DHA) concentrations in the airways' mucosa, hinting that production of protectin D1 and D-resolvins might be impaired as well (Freedman et al., 2004). It was also found that in severe asthma the expression of ALX/FPR2, the receptor binding resolvin D1 and LXA4, is reduced on peripheral blood Eos and neutrophils and increased on BAL macrophages and neutrophils and peripheral blood natural killer (NK) cells (Planaguma et al., 2008; Barnig et al., 2013; Ricklefs et al., 2017). This evidence would indicate that SPMs and their pathways might be a good candidate for detecting severe asthma and the consequent defective resolution.

A Potential New Marker: sCD48

CD48 is an activating receptor expressed on immune cells which exists in a membrane-bound form and a soluble one (sCD48) (Smith et al., 1997). CD48 on mast cells was found to interact with CD244 on Eos, initiating a cross-talk with marked pro-inflammatory outcomes in AI, the Allergic Effector Unit (Elishmereni et al., 2013). The expression of CD48 was found to be increased in Eos from peripheral blood and nasal polyps of mild asthmatic patients (Munitz et al., 2006a) and on NK cells, B-cells and T-cells of severe asthmatic patients (Gangwar et al., 2017). The levels of CD48 soluble form, sCD48, were higher in the serum of mild asthmatic patients and reduced in moderate and severe asthma (Gangwar et al., 2017). Interestingly, sCD48 levels in asthmatic patients did not correlate with Th2 inflammation markers, and this prompted the hypothesis that its expression might be linked to a broader role in inflammatory processes rather than specific AI (Breuer et al., 2018). Therefore, CD48 might be a good candidate as a biomarker for different degrees of asthma severity.

BIOMARKERS FOR ATOPIC DERMATITIS

Atopic dermatitis (AD) is among the most common inflammatory skin diseases (Nomura et al., 2020). The lack of a proper therapeutic strategy against AD has rendered this disease a significant socioeconomic burden worldwide, with higher prevalence amongst children (Barbarot et al., 2018). In AD there is increasing evidence pointing to a high degree of heterogeneity in clinical manifestations and molecular characteristics, advancing the concepts of endotypes/subtypes also for AD (Bakker et al., 2020). As with asthma, treatments for AD are moving towards the concept of personalized medicine, mostly due to the heterogeneity of the disease. This is most important, since AD is still dealt with the "one-size-fits-all" approach, which greatly limits the effectiveness of the treatment (Bieber et al., 2017).

AD Subtypes

Over the years, the characterization of AD has been significantly elucidated thereby shedding light on the complexity of this disease. This led to the classification of AD manifestations into different subtypes, namely age-related features, severity of the disease, age of onset and ethnicity according to skin condition, presence of lesions, and underlying inflammation (Bieber et al., 2017).

This classification is mainly based on the severity and the extension of the lesions and of the skin conditions, and it employs diagnostic scores such as the SCORAD or Eczema Area and Severity Index (Bieber et al., 2017). However, the underlying inflammatory response in the patients is also taken into consideration and used to define the disease characteristics. The immunological profile of AD patients shows a marked Th2 inflammation in all the subtypes, as shown by frequencies of IL-13^+ and IL-4^+ T-cells (Esaki et al., 2016b), while Th22 inflammation increased from infancy to adulthood, as shown by high levels of IL-22 in adult AD in comparison to infancy AD (Czarnowicki et al., 2020). In childhood AD, Th17 and Th9 responses were found, as demonstrated by the higher levels of cytokines such as IL-17A, IL-19 and IL-9, respectively (Esaki et al., 2016a). This immunological response might change according to the ethnicity of the patients. Asian patients present with increased Th17/Th22 inflammation, shown by the increased skin thickness and Th17/22 markers expression in skin and blood, with "psoriasis-like" manifestations (Noda et al., 2015). On the other hand, African Americans displayed increased Th22 response and skin barrier defects, while Caucasian patients showed induction of Th22, Th17 and Th1 inflammation, with reduced production of skin barrier

proteins (Nomura et al., 2020). In all ethnicities the Th2 response was always present.

This evidence shows that AD heterogeneity comprises many factors, that complicate the diagnosis and the consequent treatment. Therefore, as for asthma, also in AD biomarkers have been proposed to facilitate the definition of the disease severity.

AD Biomarkers

In contrast to what was seen in asthma, there is a general lack of suitable biomarkers for AD, mostly due to the difficulties inherent to sample retrieval. Indeed, most of the existing knowledge regarding AD biomarkers is obtained from studies performed on skin biopsies, which is an invasive and potentially dangerous method especially for infants. Therefore, new sampling methods are being used, such as skin tape-stripping, for following both the disease and the treatments (Castelo-Soccio, 2019; Guttman-Yassky et al., 2019). Another source of samples for AD biomarkers analysis is the serum of the patients (Ungar et al., 2017). Other sampling methods, less invasive, are dried blood spots (DBS), consisting in droplets of blood collected via a capillary and absorbed on a cellulose layer. DBS are then eluted via an adequate buffer and processed for biomarkers analysis. This technique is minimally painful and easy to process (J. L. Thijs et al., 2019). Another less invasive source of samples is saliva, mainly due to the possibility of blood biomarkers diffusing into the salivary glands (Thijs J. et al., 2015). Biomarkers for AD in the skin are generally measured via their mRNA levels, while serum biomarkers can be analyzed also via ELISA (Ungar et al., 2017; Guttman-Yassky et al., 2019). Identification of potential patients before symptoms appearance involves evaluation of the skin barrier functionality, via epidermal water loss and expression of FLG1-2, encoding for the proteins filaggrin 1 and 2, or other structural skin proteins (Margolis et al., 2014; Bager et al., 2016). The stratification of patients is done also according to the underlying immunological response. AD is mainly a Th2-driven disease, characterized by expression of IL-4, IL-5 and IL-13, and the levels of these cytokines can also predict the outcomes of anti-IL-4, anti-IL-5 and anti-IL-13 biologics therapies (Bakker et al., 2020). Where the phenotype is more Th17-dominant, the levels of IL-17 are evaluated, and might be a good marker for following anti-IL-17-directed approaches (Bieber et al., 2017). In patients showing upregulation of the Th22 response, IL-22 is the most evaluated marker for disease severity, and its levels were also shown to be predictive of fezakinumab (anti-IL-22) administration outcomes (Brunner et al., 2019). Other markers for AD severity include ECP, TSLP, β-defensin 1, eotaxin, RANTES, CCL17-22-27, the latter allowing T-cells homing to the skin (Bieber et al., 2017; Bakker et al., 2020). Among these markers, CCL17 was the one showing the highest correlation with AD severity, although its levels are generally variable with AD heterogeneity and underlying inflammatory pathways (Landheer et al., 2014; Thijs J. L. et al., 2015). Sensitization to the allergen is measured via total and specific IgE, however the IgE profile is subject to great variation among the population. Therefore, it would be more useful to examine the ratio between specific and total IgE (Bieber et al., 2017).

New Potential Biomarkers

Adipokines

In a recent study, the levels of serum adipokines were evaluated and related to the disease features. Two adipokines, adiponectin and resistin, showed lower levels in AD patients and an inverse proportionality trend with the severity of the disease. On the other hand, leptin levels were increased in AD patients, but did not correlate with disease severity. No correlation between adipokines' levels and patients' characteristics (age, gender, BMI) was found (Jaworek et al., 2020). Thus, although requiring more studies, adipokines might be a new interesting and more specific set of biomarkers linked to AD severity.

CD300a

CD300a is an inhibitory receptor expressed on the surface of several immune cells, and its role in downregulating AI has been extensively demonstrated (Bachelet et al., 2005; Bachelet et al., 2006; Munitz et al., 2006b). It was recently published that total CD300a expression is increased in lesional AD skin and specifically on Eos, and that its expression positively correlated with hypoxic conditions and angiogenesis in AD skin (Karra et al., 2019). Moreover, CD300a expression was significantly increased on B-cells from AD patients and decreased on circulating NK cells (Karra et al., 2019). Interestingly, CD300a expression was not increased in non-lesional AD skin [(Karra et al., 2019) supporting information)], hinting that this receptor might be a marker for severe forms of the disease.

CD48

CD48 surface levels were found to be significantly decreased in peripheral blood from mild/moderate/severe AD patients, and on Eos, neutrophils, monocytes, basophils, NK cells, T- and B-cells (Minai-Fleminger et al., 2014). However, its expression was significantly increased on Eos in biopsies from lesional AD skin (Minai-Fleminger et al., 2014). It was hypothesized that this differential CD48 expression might be the result of CD48 sensitivity to local stimuli rather than systemic ones (Minai-Fleminger et al., 2014). Thus, CD48 might provide information regarding local inflammation in AD lesional skin.

Skin Microbiome

The role of secondary infections in AD, especially from bacteria such as *Staphylococcus aureus*, normally residing on the skin, is well characterized (Weidinger and Novak, 2016). A recent study has shown that AD skin presents with dysbiosis in comparison to healthy controls, with prevalence of *Staphylococcus aureus* and reduction in anaerobic bacteria species, correlating with disease severity (Fyhrquist et al., 2019). In another study, the skin microbiome composition was employed to divide patients in "dermotypes", each one with distinct bacterial genera prevalence and metabolic profiles (Tay et al., 2020). Among the dermotypes, the "B" one presented with higher Th2-specific mediators, worsened symptomatology and increased possibility to develop other atopic diseases (Tay et al., 2020).

This evidence adds another level of characterization of AD, which might aid in stratification of the patients and evaluation of biomarkers.

CONCLUDING REMARKS

Since the emergence of the concept of personalized medicine, it has become clear that the "one-size-fits-all" approach for allergic diseases is not adequate to treat the high heterogeneity of patients. Thus, the search for biomarkers for predicting the occurrence and the outcomes of the disease was prompted. Despite the vast amount of research conducted, there is still a need for less invasive sampling techniques and more sensitive markers.

New techniques include the use of -omics technology, such as transcriptomics, proteomics and metabolomics, to create a detailed profile of the asthma features. One of the latest applications is the metabolic profiling of the breath of asthma patients before and after ICS treatment (Ferraro et al., 2020). Moreover, the aim of the ongoing SMART clinical trial (NCT04194814), started in 2019, is to test new non-invasive methods to evaluate biomarkers for skin structure and function variations.

In conclusion, biomarkers for asthma and AD provide useful tools in the diagnosis of the disease and the prediction of the symptoms' occurrence and therapeutical responses/outcomes. Novel methodologies for both sampling and analysis are now being evaluated. In time, this might result in better analytic strategies that would benefit both the patient and the clinician in terms of non-invasiveness, reliability and specificity of the marker, in order to design the best therapeutical approach for each patient.

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REVIEW
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Machine Learning Techniques for Personalised Medicine Approaches in Immune-Mediated Chronic Inflammatory Diseases: Applications and Challenges

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In the past decade, the emergence of machine learning (ML) applications has led to significant advances towards implementation of personalised medicine approaches for improved health care, due to the exceptional performance of ML models when utilising complex big data. The immune-mediated chronic inflammatory diseases are a group of complex disorders associated with dysregulated immune responses resulting in inflammation affecting various organs and systems. The heterogeneous nature of these diseases poses great challenges for tailored disease management and addressing unmet patient needs. Applying novel ML techniques to the clinical study of chronic inflammatory diseases shows promising results and great potential for precision medicine applications in clinical research and practice. In this review, we highlight the clinical applications of various ML techniques for prediction, diagnosis and prognosis of autoimmune rheumatic diseases, inflammatory bowel disease, autoimmune chronic kidney disease, and multiple sclerosis, as well as ML applications for patient stratification and treatment selection. We highlight the use of ML in drug development, including target identification, validation and drug repurposing, as well as challenges related to data interpretation and validation, and ethical concerns related to the use of artificial intelligence in clinical research.

Keywords: machine learning, autoimmune disease, personalised medicine, biomarker, omics

INTRODUCTION

Machine learning (ML) is one subset of artificial intelligence (AI) that aims to build analytical models by learning from existing data. The concept of AI and ML can be traced back to the mid-20th century when building a "machine that can learn from experience" was proposed by mathematician Alan Turing (Turing, 1995). After decades of incremental development and technological innovation, ML has emerged as a powerful discipline for a wide range of scientific research and industrial applications, with a particular strength in discovering patterns in complex, high dimensional data and examining non-linear relationships. In recent years, substantial clinical breakthroughs using ML applications have been made including disease prevention, diagnosis, prognosis, drug

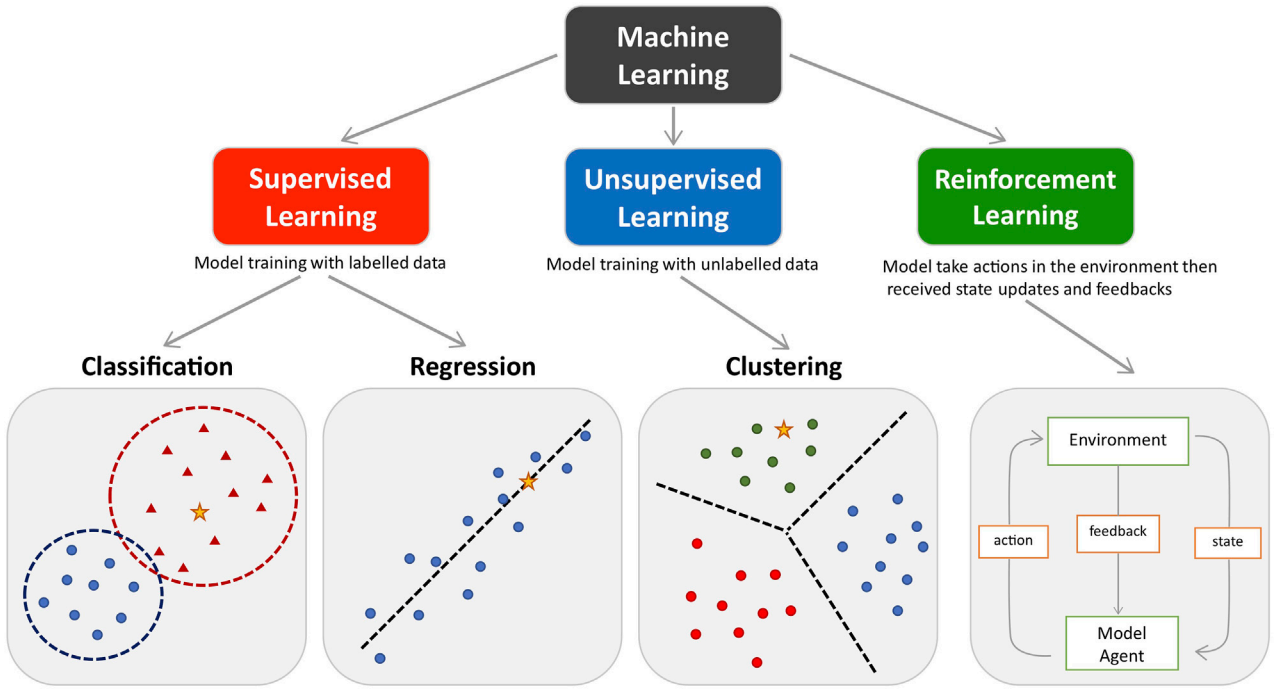

FIGURE 1 | The main types of machine learning. Main approaches include classification and regression under the supervised learning and clustering under the unsupervised learning. Reinforcement learning enhance the model performance by interacting with environment. Coloured dots and triangles represent the training data. Yellow stars represent the new data which can be predicted by the trained model.

discovery and clinical trial design (Stafford et al., 2020; MacEachern and Forkert, 2021). Indeed, the rapid expansion in the availability of patient data has now placed ML under the spotlight for developing data-oriented precision medicine approaches. Immune-mediated inflammatory diseases, such as autoimmune rheumatic diseases (ARDs), inflammatory bowel disease (IBD), immune mediated chronic kidney disease (CKD) and multiple sclerosis (MS), comprise a large group of complex, multifactorial conditions associated with chronic inflammation triggered by dysregulated immune responses. These diseases are highly heterogeneous in presentation, commonly involving multi-organs and systems, and therefore are characterised by complex pathogenic mechanisms and highly variable response to therapies. Thus, applying advanced ML techniques to the clinical study of immune-mediated inflammatory diseases could help develop personalised medicine approaches and improved disease management. In this review, ML applications in clinical research are highlighted and the key challenges and limitations of applying ML towards the goal of personalised medicine in various immune-mediated chronic inflammatory diseases are discussed.

Types of Machine Learning

ML approaches can be generally divided into three types: supervised, unsupervised and reinforcement learning, tailored for distinct investigation purposes (**Figure 1** and *Glossary*). Supervised learning algorithms investigate relationships between predictive variables and outcome from labelled training datasets and apply the learned rule to establish a model for classifying new data (Russell et al., 2010). Classification and regression are two major approaches in supervised learning, where the classification model aims to predict category outcome (e.g., diagnosis given by clinician) and the regression model aims to predict a continuous outcome (e.g., disease activity score). The application of supervised learning models is crucial for biomarker identification in precision diagnostic and therapeutic decision making, as well as predicting disease prognosis. Conversely, unsupervised learning algorithms are applied to uncover hidden patterns in training data without labels. Clustering approaches within unsupervised learning, including hierarchical clustering, K-means clustering and Gaussian mixture models, are the most popular techniques for assembling data into previously ambiguous bundles. Unsupervised clustering approaches form the decisive component in most patient stratification studies and in identifying disease subtypes (Mossotto et al., 2017; Orange et al., 2018; Robinson et al., 2020; Martin-Gutierrez et al., 2021). Finally, reinforcement learning is scripted to sequentially self-correct from environmental feedback (positive or negative) and therefore improve the overall model function without having labelled data (Kaelbling et al., 1996). While the application of reinforcement learning is less prevalent in clinical research compared to supervised and unsupervised learning, the value of reinforcement learning in clinical trial design is highlighted in numerous studies (Padmanabhan et al., 2015; Yauney and Shah, 2018; Ribba et al., 2020). Moreover, deep

learning, inspired by the biological neural communication networks in the brain, is a noteworthy subset of ML algorithms for processing data and extracting patterns that are used for decision-making. Deep learning can be designed as a supervised, unsupervised or reinforcement model, which allows it to handle a variety of tasks. Popular deep learning algorithms such as recurrent neural networks (RNN) and convolutional neural networks (CNN) are powerful tools in the field of computer vision, where medical imaging recognition is widely studied for disease diagnosis (Le et al., 2009), prognosis (Klang et al., 2020) and subtypes identification (Suzuki, 2017; Jaber et al., 2020).

Data Types

The tremendous expansion of patient-derived data accounts for the popularity of ML approaches in the quest for precision medicine. Extensive types of patient data are collected as part of electronic health records (EHR) (e.g., patient demographic data, routine clinical and serological measurements, imaging data) and clinical research (e.g., omics data).

Data characteristics, such as universality and potential applicability for developing effective precision medicine approaches, facilitate ML-based clinical studies. Electronic medical records (EMR) data are the most systematically collected patient data with standardised format and are frequently applied in clinical ML applications because they are relatively accessible and easy-to-implement. EMRs are digital data compiled by healthcare systems, they contain longitudinal information from individuals, such as medical history, current diagnoses, medication, disease activity and other clinical measurements collected at a particular clinical visit. EHRs contain information beyond EMRs, including cumulative laboratory and imaging data available for a certain patient as well as information about their overall health from all the clinicians involved in their care. Applying ML to data from EMR/EHRs is a major area of interest within the field of personalised diagnosis and treatment (Landi et al., 2020).

Medical imaging including magnetic resonance imaging (MRI), computed tomography, nuclear imaging, x-ray, electroencephalography and ultrasound etc., are all techniques with standardised imaging acquisition protocols. These data are predominantly analysed by deep learning algorithms, which are the most suitable due to their strength and competence in analysing the complex detail present in medical images. Deep learning techniques have shown particular progress in precision oncology including early diagnosis, identifying cancer subtypes, early detection of metastasis and aiding clinical decision-making (Liu et al., 2019; Munir et al., 2019; Tandel et al., 2019).

There are various applications of ML techniques in radiology, from automatization of routine tasks usually performed by radiologists and clinicians requesting various investigations, such as assessment of imaging appropriateness, creating study protocols to improve image quality and minimise radiation, and standardisation of the way radiology studies are reported (Lakhani et al., 2018).

Although the majority of ML applications in radiology are not specific for use in immune-mediated chronic inflammatory conditions, which are the focus of our review, various ML algorithms have been implemented in clinical practice, such as *medical image segmentation* (Cooper et al., 1998) which can be applied to various types of imaging (e.g., brain, spine, lung, liver, kidney, colon); *medical image registration* (e.g. integration of various complementary imaging modalities or time series to facilitate diagnosis); *computer-aided detection and diagnosis* (Doi, 2007) (e.g., mammography, CT colonography, and CT lung for detection of nodules which assist clinicians in diagnosis by reducing reading time and improving the sensitivity of the detection of pathological findings); *brain function/activity analysis and diagnosis of neurological conditions using functional MR (fMR) images* (Pereira et al., 2009) (to facilitate the non-invasive interpretation of high dimensional data related to the brain function); *content based image retrieval* systems which enables searching for digital images in large databases based on the contents of the image to facilitate diagnosis by comparing images with similar features or from previously-confirmed cases with the same diagnosis; and *text analysis of radiology reports* (Dreyer et al., 2005) using natural language processing (NLP) and natural language understanding (NLU) (Wang and Summers, 2012).

Biomarker discovery and application is a main focus in modern-day clinical research, where quantified molecular signatures are used as indicators for predicting different aspects of certain diseases. Compared to traditional evaluation of patients by direct clinical observations of the disease presentation, multiple biomarker panels from high dimensional data measured by state-of-the-art technology allow researchers to pinpoint disease endotypes from a wide spectrum of clinical presentations and could be particularly important for precision medicine in complex human diseases. For disease diagnosis, biomarkers that can be routinely collected by cheap and easily accessible approaches are preferable since periodic assessment is crucial for disease detection and early intervention of high-risk populations. Alternatively, prognostic biomarkers for predicting associations with mortality, disease progression, and more active disease, usually involve disease specific investigations, including analysis of blood (Robinson et al., 2020; Coelewij et al., 2021), urine (Glazyrin et al., 2020), cerebrospinal fluid (Toscano and Patti, 2021), tears (Torok et al., 2013) and even breath (Sola Martínez et al., 2020), as well as routinely collected imaging data (Ciurtin et al., 2019). Omics analysis of such biological material, including metabolomics, proteomics, RNA-sequencing (so-called "big data") and autoantibody data are used to study diagnosis and prediction of disease activity in inflammatory chronic diseases (Teruel et al., 2017; Imhann et al., 2019). Furthermore, digital clinical data extracted from EHR can potentially provide digital biomarkers for disease diagnosis and risk prediction (Wu et al., 2017). With the power of deep learning, biomarkers extracted from imaging data have already extended the accuracy of human decision-making (Liu et al., 2019). However, the expensive operating cost, the invasiveness of certain imaging approaches and the demand of a relatively large data size to generate meaningful outcomes from ML models are major drawbacks for applying imaging biomarkers in ML-based clinical research. For predicting treatment response such as treatment resistance and recurrence risk in inflammatory diseases, genetic,

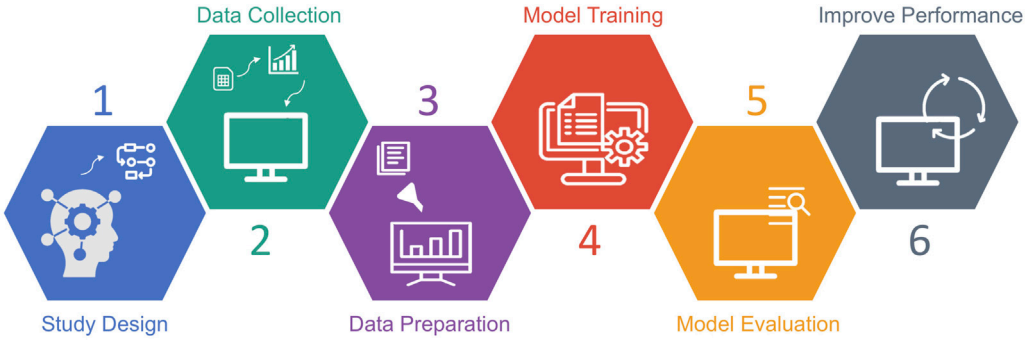

FIGURE 2 | Workflow of machine learning study.

serological and immunological biomarkers and clinical phenotyping are frequently applied (Bek et al., 2016; Figgett et al., 2019; Waddington et al., 2020).

Workflow for Building Machine Learning Models

To be intelligent and provide new solutions for intractable clinical needs, ML needs to learn and improve from the given data and apply it in a dynamic environment. Essential steps involved in building a ML model include study design, data collection, data preparation, model training, model evaluation and performance improvement (**Figure 2**). Before the actual model training, a thoughtful study design that answers key questions including what the unmet clinical need is, what types of data need to be collected and applied, what types of ML are suitable to address the study aims etc., are critical for building effective ML models with suitable clinical value. Gathering data is the first and most important step of any ML approach, since making inferences from a given sample is the core task of ML. The quantity and quality of the collected samples determine whether the model is effective and representative when applied in practice. Subsequently, the data preparation process prunes the raw data into a specific format. Models are constructed using the training dataset and further evaluated using the validation/testing dataset. The model validation includes internal validation (e.g., k-fold cross-validation) and external validation using an external cohort. Finally, model performance is enhanced by repeatedly undergoing model training and evaluation processes until the performance is optimal.

APPLICATIONS

Machine Learning Applications in Immune Mediated Inflammatory Disease: Prediction, Diagnosis and Prognosis

One of the main strengths of ML is the ability to analyse data with many variables and perform biomarker selection, which could contribute to precision diagnosis and prognosis. Traditional analysis techniques tend to examine linear relationships between individual variables and outcomes and are often heavily dependent on existing knowledge, which is often inefficient and short-sighted when dealing with datasets with overwhelmingly high dimensions, as is the case with omics data. In contrast, ML approaches can sufficiently handle a large number of variables in the dataset and can also quantify and rank the variable importance in model training. For example, the "mean decrease in Gini" in the random forest model measures the average (mean) of the total decrease in node impurity of variable, weighted by the proportion of samples reaching that node in each individual decision tree in the random forest; thus, a higher "mean decrease in Gini" implies a greater contribution of a variable to the overall model performance (see *Glossary*). ML methods allow a robust biomarker selection process, enabling researchers to quickly screen out and combine the most relevant markers for more comprehensive decision-making. Effective biomarker selection has been applied extensively in diseases with a strong genetic determinant such as cancer (Henry and Hayes, 2012). However, this is more challenging in multifactorial diseases with substantial environmental susceptibility factors such as autoimmune inflammatory diseases.

Machine Learning for Diagnosis

There are multiple examples in the literature where predictive ML models have been used to identify diagnostic biomarkers in immune mediated inflammatory diseases (**Table 1**) (Seyed Tabib et al., 2020; Stafford et al., 2020). For example, ML techniques applied to proteomics have differentiated between immune-mediated CKD and other causes of CKD (Glazyrin et al., 2020). In this study, plasma proteomics data from 131 subjects balanced across CKD disease patient subtypes (diabetic nephropathy, glomerulonephritis and hypertensive nephropathy) and healthy controls were analysed. Principle component analysis (PCA) selected 175 relevant protein predictors, which were individually assessed using conventional statistical methods, but no significant differences were identified between the groups. However, using the K-nearest neighbours ML model, the CKD disease group was discriminated from the healthy group with a 97.8% accuracy, and patients with diabetic nephropathy were separated from glomerulonephritis

TABLE 1 | Examples of machine learning application in precision diagnosis and prognosis of inflammatory diseases.

ML algorithms	Type of data	Sample sizes	Applications	References
		Applications in Disease Diagnosis		
kNN, LR, SVM, DT, PCA	Plasma and urine proteomics	131 plasma and 47 urine samples from CKD patients	Proteomics-based ML approach was developed as differential diagnosis tool of early state CKD.	Glazyrin et al. (2020)
RF	Immunophenotyping	72 JIA and 43 healthy controls	ML methods applied to identify JIA patients from healthy controls by immune profile	Van Nieuwenhove et al. (2019)
SVM, RF, kNN, NB	fMRI connectivity matrix	41 neuropsychiatric SLE patients and 31 healthy controls	ML classifiers applied for Neuropsychiatric SLE patients using resting-state fMRI functional connectivity	Simos et al. (2019)
unsupervised surrogate assisted feature selection (SAFE), NLP, LR	Electronic Health Records	114 definite SLE, 49 probable SLE, 237 Non-SLE patients	ML algorithms were applied to identify lupus patients in electronic health records and validated the performance of existing rule-based algorithms	Jorge et al. (2019)
AdaBoost	Electronic Health Records	583 SLE, 16174 non-SLE patients	ML model trained with noisy labelled electronic health records are used for heterogenous lupus identification	Murray et al. (2018)
		Applications in Disease Prognosis		
Elastic generalized linear model (GLM), KNN, RF	Whole blood gene expression data	156 SLE (82 active; 74 inactive) patients	Supervised ML approaches were applied to predict lupus disease activity using gene expression data	Kegerreis et al. (2019)
Multinomial LR	Laboratory measurements and demographics	286 SLE with 5,680 visits	Screening ML models to identify high disease activity SLE patients using simple demographic and laboratory measurements	Hoi et al. (2021)
RNNs	Clinical and laboratory measurements	132 SLE patients with no baseline chronic damage (in the 2 years follow up, 38/132 developed chronic damage)	ML algorithms were used to predict the risk of chronic damage of SLE patients using longitudinal clinical and laboratory measurements	Ceccarelli et al. (2017)
RF, SVM, KNN, AdaBoost, RNNs	Clinical records	1,624 MS patients (follow up visits in 180, 360 and 720 days)	Supervised ML algorithms were applied to predict disease course of MS patients using longitudinal clinical records	Seccia et al. (2020)
Elastic net (GLM)	Quantitative measurements from routine clinical tests	3,515 young and asymptomatic individuals	General linear model was applied to predict subclinical atherosclerosis risk in young and asymptomatic individuals using longitudinal quantitative laboratory measurements and routine clinical tests	Sánchez-Cabo et al. (2020)
RF, LR with and without interaction, SVM, DT	Serum metabolomics data	80 female SLE patients	Supervised ML classifiers were applied to predict subclinical atherosclerosis in SLE patients using serum metabolomics data	Coelewij et al. (2021)

Abbreviation: ML, Machine learning; PCA, principal component analysis; LR, logistic regression model; GLM, generalized linear model; SVM, support vector machine; GB, gradient boosting; XGBoost, extreme gradient boosting; RF, random forest; DT, decision tree; ET, extremely random trees; GBDT, gradient boosting decision tree; NB, naïve Bayes; NN, neural network; CNN, convolutional neural networks; RNNs, recurrent neural networks; DL, deep learning; kNN, k-nearest neighbours; NLP, natural language processing; CKD, chronic kidney disease; JIA, juvenile idiopathic arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis.

patients with a classification accuracy of over 96%. A similar approach was performed with proteomic analysis of 47 urine samples, which separated healthy controls from CKD disease with high performance but failed to effectively discriminate within CKD disease subtypes (Glazyrin et al., 2020). However, the extremely small dataset in the urine study (eight samples in the smallest group) greatly limited the power of the ML model as well as giving an unreliable model performance, due to the concern of model overfitting the training data (to be discussed in a later section). Although many ML approaches can deal with the classification of multiple groups, a decrease in robustness for most models is inevitable when the number of classes increases. To overcome this, the above study proposed a two-stage differential diagnosis; the urine-based ML model for separating hypertensive nephropathy and healthy control samples from patients with CKD, to be followed by the plasma-based model to separate patients with glomerulonephritis and diabetic nephropathy. Thus, this study provides a potential early diagnosis strategy using proteomics-based ML-models coupled with the ability to differentiate between disease subtypes. This could decrease the use of invasive kidney biopsies, although further external validation on a large cohort is essential.

Researchers have also explored precision diagnosis of juvenile idiopathic arthritis (JIA), a heterogeneous autoimmune disease, using immune-based ML approaches (Van Nieuwenhove et al., 2019). Immunophenotyping data of 72 JIA patients and 43 age-matched healthy controls were used as predictors for the classification model (random forest). After optimisation and 10-fold cross-validation, the random forest model had high performance with an area under the curve (AUC) of 0.90

when discriminating JIA from healthy using all 42 immune cell subtypes. iNKT cell subtype was the variable that contributed most to the random forest model (assessed by mean decrease in Gini), and was used to build a univariable (iNKT cell only) model which had an AUC of 0.91. However, after removing iNKT cells from the model (keeping all other predictors), the model maintained a good performance (AUC = 0.86). The order of the variable ranking also remained the same in models with and without iNKT cells. These results suggested that the contribution of iNKT cells to JIA pathogenesis may not be the most important despite being the top ranked variable by the random forest model. The study illustrates the power of ML analysis in explaining biological function and the potential clinical application in precision diagnosis of JIA.

In a study of patients with neuropsychiatric SLE (Simos et al., 2019), researchers applied a ML model to enhance current neuropsychiatric SLE diagnosis approaches based on resting-state functional connectivity MRI (fMRI) imaging data of the brain. ML classifiers, including random forest, support vector machine, naïve Bayes and k-nearest neighbours were trained by the fMRI connectivity matrix derived from fMRI images of the brain network of 41 neuropsychiatric SLE patients and 31 healthy controls. The support vector machine model achieved the best performance, identifying neuropsychiatric SLE patients with and AUC 0.75, validated by 5-fold cross-validation. This model also indicated that the frontoparietal brain region contributed most to the performance. However, the model performance is not outstanding for practical use in diagnosis, therefore testing a larger cohort for model training and performing appropriate external validation in future studies could potentially elevate the model quality and help build a neuropsychiatric SLE classification pipeline.

A number of studies have begun to examine the application of ML techniques to the diagnosis of complex autoimmune diseases using EHR and EMR data (Murray et al., 2018; Jorge et al., 2019). In a previous study by Jorge et al. (2019), ML algorithms were able to identify patients with systemic lupus erythematosus (SLE), a complex disease whose diagnosis requires multiple criteria, including clinical presentation, history of symptoms and, laboratory data. Patients with an international classification of disease (ICD) code that suggested a possible diagnosis of SLE (without fulfilling the criteria for classification as having SLE) were included in the model training. Selected EMR records were then defined, and the corresponding patients were assessed by rheumatologists using clinical expertise and validated SLE classification criteria, and categorised as either definite SLE, probable SLE and non-SLE. A novel ML approach combined the rule-based and natural language processing (NLP) algorithms (Teller, 2000) to identify SLE patients using EHR data (including laboratory measurements, medications and disease history). The model achieved an overall good performance (AUC = 0.909) with a 92% positive prediction rate when classifying SLE (definite and probable) from non-SLE cases. Although the performance of ML models was not improved compared to the rule-based methods, the combined method demonstrated a good performance on both internal and external validation. This is particularly important for developing a portable and universal pipeline for identifying SLE patients based on medical records and implementing into a healthcare system and could provide a model for classifying complex diseases such as SLE.

In another study using EHR data to identify SLE patients (Murray et al., 2018), an ensemble algorithm (AdaBoost learners, EasyEnsemble (Liu et al., 2009)) was applied to an imbalanced dataset (derived from 583 SLE, and 16174 non-SLE individual patient EHR). A high model performance was achieved (AUC 0.97) and maintained in the testing dataset (AUC 0.94), where definitions of SLE were validated by two rheumatologists using "strict" and "inclusive" terms respectively.

Similar studies have applied EMR data to classify patients with rheumatoid arthritis (RA) (Liao et al., 2010) and IBD (Ananthakrishnan et al., 2013), as well as to identify patient subsets. For example, a study used EHR to identify methotrexate-induced liver toxicity in RA patients (Lin et al., 2015). A logistic regression model was used to classify cases as having or not methotrexate induced liver toxicity, with a 0.756 positive predictive value. Moreover, EHR-based ML models can be used to screen for genetic disorders with long term health effects such as familial hypercholesterolemia, which can remain largely undiagnosed due to the strict privacy rules for universal screening in some areas. A "random forest" -based ML algorithm (FIND FH) developed by Myers and colleagues (Myers et al., 2019) identified individuals with a high chance of having familial hypercholesterolemia using information available on external healthcare system databases. Samples from the identified individuals at risk for FH were further validated by experts with a precision ranging from 77 to 87%, showing that EHR-based ML models could be a promising preselection tool for identifying patients at risk for genetic conditions without universal screening.

Machine Learning in Predicting Disease Prognosis

ML classification models can also be applied in disease activity prediction of complex autoimmune diseases (**Table 1**). This has been attempted in several ways as can be demonstrated in SLE. In a study using whole blood gene expression data, SLE disease activity was predicted by ML classifiers (Kegerreis et al., 2019). The gene expression and module enrichment data of 156 SLE patients from three datasets were included and stratified for disease activity using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI); active disease SLEDAI≥6 and inactive disease SLEDAI<6. Interestingly, both conventional gene differential expression analysis and unsupervised clustering methods (hierarchical clustering) failed to distinguish SLE patients based on their disease activity alone, potentially due to the heterogeneous and complex nature of the disease. Therefore, supervised ML classifiers including random forest, k-nearest neighbours and generalized linear models were used to separate patients with active versus inactive disease. The random forest classifier scored the highest performance with a peak accuracy of 83% when using raw gene expression data as a predictor. However, the model performance varied dramatically when validated by datasets using different technical settings. When gene modules were used as model predictors, the performance of the random forest classifier was stable at

around 70% accuracy regardless of the validation approach. The mean decrease in Gini impurity from the random forest model indicated an important role for $CD14^+$ monocytes in SLE patients with active disease. Although models trained by gene expression data remain challenging for implementation in the clinical setting from the point of view of feasibility and cost-effectiveness, the gene expression features identified between active and inactive groups of patients may boost the understanding of SLE pathogenesis.

Another study attempted to identify SLE patients with high disease activity using ML algorithms without making use of the validated disease activity score usually implemented in routine practice (SLEDAI) (Hoi et al., 2021). The longitudinal data of 286 SLE patients (median follow up 5.1 years, a total of 5,680 visits) including measurements of High Disease Activity (HDA), defined as SLEDAI-2K≥10, 16 laboratory and three demographic parameters (age, sex, and ethnicity) were used to build a multinomial logistic regression model. After screening a total of 2^{16} models with different variable settings for optimisation purposes, the final model including seven laboratory variables and three demographic variables identified with 88.6% accuracy whether a certain SLE patient had HDA or not. The model training used data from all visits, irrespective of their time point and this limited the possibility of using certain earlier time-points to predict later disease development status. The study shows the possibility of using a limited amount of routinely available laboratory measurements and demographics to select SLE patients with HDA, which could help the early identification of SLE patients likely to require treatment escalation after testing the model in a clinical setting.

Another study accurately predicted chronic damage in SLE with the aim to improve disease management (Ceccarelli et al., 2017). 413 SLE patients were assessed for chronic damage evaluated by the validated SLICC/ACR Damage Index (SDI) (Gladman et al., 1996), which includes longitudinal measurements of damage potentially acquired within 12 organ systems. Supervised recurrent neural network (RNN) which is a class of artificial neural network (see *Glossary*) was employed to classify patients without chronic damage at baseline but who developed damage in the following 2 years versus those who did not develop chronic damage. Clinical data including demographics, diagnosis date, co-morbidities and medical history, and laboratory data including important markers of SLE were used as predictors for RNN model training. The RNN model uses the all the longitudinal time point (≥5 visits for each patient) of chronic damage measurement as the sequential input, then processes the network through the hidden layer (layers in between) until connecting the output layer, which generates the prediction results (see *Glossary*). To avoid overfitting, an early stopping technique (stop when AUC reaches 0.95) and 8-fold cross-validation were applied. The model performance was stable at AUC (0.77) for predicting a chronic damage-developing group.

Similar studies have also been described in patients with MS. Seccia and colleagues applied supervised ML algorithms to predict disease progression of MS and potentially provide treatment decision support (Seccia et al., 2020). Four common ML algorithms (random forest, support vector machine, k-nearest neighbours and AdaBoost) were employed to identify whether patients with MS will evolve from the initial Relapsing-Remitting (RR) phase to the Secondary Progressive (SP) phase over 180, 360 and 720 days using real-world clinical data. After model optimisation, the prediction accuracy of random forest, support vector machine, and AdaBoost models had similar performances around 85% for 180-, 360- and 720 days progression prediction. Due to the nature of MS evolution, the sample size of transitioning (SP) patients is usually significantly smaller than the non-transitioning (RR) patients. This extremely imbalanced data limited the overall performance of the model and could be improved by a larger study cohort with more balanced data and external validation. Moreover, a more integrated and comprehensive approach combing results from all the high performing models could improve the overall prediction.

The classification and biomarker selection properties of ML algorithms can also help to predict the prognosis of diseases with a long asymptomatic phase. In a recent study of atherosclerosis, Sánchez-Cabo and colleagues applied ML to predict cardiovascular risk in asymptomatic individuals (Sánchez-Cabo et al., 2020). Non-invasive imaging such as computerised tomography and vascular ultrasound can help to assess cardiovascular risk but are only recommended in clinical practice after evaluating traditional risk factors such as serum cholesterol levels, which could underestimate the long-term cardiovascular risk in asymptomatic individuals. In this study, ML models were built based on 3,515 individuals with 115 quantitative predictors collected from routine clinical tests. Baseline imaging was used to classify samples into four groups (no disease, focal disease, intermediate disease, generalized disease) based on the detection of subclinical atherosclerosis. The "no disease" and "generalized disease" classes were used to build up an elastic net model (penalized linear regression model) (see *Glossary*) using all predictors. After variable selection from the model, a refined model with 12 predictors was employed. The refined elastic net model significantly outperformed the traditional cardiovascular risk assessment scores in predicting generalized subclinical atherosclerosis and the risk of progression in 3 years. Notably, this model improved the false-negative prediction rate meaning that fewer high-risk individuals were mis-classified in the "no disease" group.

In a recent study of SLE (Coelewij et al., 2021), researchers attempted to predict subclinical atherosclerosis in SLE patients using serum metabolomics data. 228 metabolites from 80 female SLE patients were quantified by nuclear magnetic resonance spectroscopy and used as predictors. Subclinical atherosclerosis status of each patient was assessed by femoral and carotid artery ultrasound scans. After pre-processing the serum metabolomics data (imputation of missing data, homology reduction and data scaling), five supervised classification models were applied to predict subclinical atherosclerosis. The logistic regression with interactions model achieved the highest classification accuracy (80%). Feature selection was performed using the top three models (random forest, logistic regression with and without interaction) in predicting subclinical atherosclerosis in SLE,

TABLE 2 | Examples of machine learning application in subtype identification, therapy selection and drug development of inflammatory diseases.

ML algorithms	Types of data	Sample sizes	Application	References
Applications in Disease Subtype Identification and Therapy Selection				
PCA, PLSDA, sPLS-DA, k-means clustering, hierarchical clustering	Whole-blood RNA sequencing data	161 SLE and 57 healthy controls	ML clustering approaches were applied to stratify SLE patients based on gene expression signatures	Figgett et al. (2019)
RF, sPLS-DA, k-means clustering	Immunophenotyping	45 SS, 29 SLE,14 patients with both conditions and 31 healthy controls	ML and statistical approaches were applied to discover shared immune profile between SS and SLE. Immune cell signatures were used to stratify patients into groups with different clinical presentation regardless of the diagnosis	Martin-Gutierrez et al. (2021)
RF, sPLS-DA, k-means clustering	Immunophenotyping	67 juvenile-onset SLE patients and 39 healthy controls	ML and statistical approaches were applied to identify juvenile-onset SLE from healthy controls using immunophenotyping data. The immune cell signatures were used to stratify patients into four groups with different clinical manifestations	Robinson et al. (2020)
XGBoost, RF, GBDT, ET and LR	Electronic Medical Record	87 JIA patients with etanercept treatment	Supervised classifiers were applied to predict the treatment efficacy of etanercept in JIA patients	Mo et al. (2020)
DT, RF, kNN, SVM, LR with and without interactions	Serum metabolites	89 MS patients with IFNβ treatment	Supervised classifiers were applied to predict the anti-drug antibody development in MS patients before and after IFNβ treatment	Waddington et al. (2020)
Applications in Drug Development				
DL (deepDTnet)	15 types of chemical, genomic, phenotypic, and cellular network profiles	732 small molecules	A DL approach was developed for novel target identification and drug repurposing using heterogeneous drug–gene–disease networks from existing drugs	Zeng et al. (2020)
Bayesian network (BANDIT)	Drug efficacies, post-treatment transcriptional responses, drug structures, reported adverse effects, bioassay results and known targets	>2,000 small molecules	A Bayesian machine learning approach was developed for novel binding target prediction using diverse data types	Madhukar et al. (2019)
Translational Network for Indication Prediction (CATNIP)	16 different drug similarity features	2,576 small molecules	ML algorithm was developed for drug repurposing using only biological and chemical information of the molecules	Gilvary et al. (2020)
DL (MathDL)	Public databases (PDBbind and ChEMBL)	17,382 protein–ligand complexes (PDBbind) and 2 million compounds (ChEMBL)	DL and algebraic topology were used to rank the attractive binding sites for SARS-CoV-2 drug development. The model identified 71 covalent bonding inhibitors for SARS-CoV-2 main protease, a favourable drug target of SARS-CoV-2	Nguyen et al. (2020)

Abbreviation: ML, Machine learning; PCA, principal component analysis; sPLS-DA, sparse partial least squares-discriminant analysis; LR, logistic regression model; SVM, support vector machine; GB, gradient boosting; XGBoost, extreme gradient boosting; RF, random forest; DT, decision tree; ET, extremely random trees; GBDT, gradient boosting decision tree; NB, naïve Bayes; NN, neural network; CNN, convolutional neural networks; RNNs, recurrent neural networks; DL, deep learning; kNN, k-nearest neighbours; NLP, natural language processing; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; JIA, juvenile idiopathic arthritis; MS, multiple sclerosis; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

where very low-density lipoprotein (VLDL) subclasses and leucine were top ranked in the ML model and were also validated by the univariate logistic regression. As SLE patients are known to be at higher risk of developing cardiovascular disease compared to age and sex-matched healthy individuals, this study revealed the possibility of using serum biomarkers to identify SLE patients with high cardiovascular disease risk early and allow adequate preventative strategies to address this risk. ML techniques have also been used for complex risk disease prediction using both genetic and nongenetic data with different levels of performance. A 7 years longitudinal study in patients with hepatitis C identified that boosted-survival-tree models were statistically superior to cross-sectional or linear models for predicting development of cirrhosis in chronic hepatitis C as a model of a disease with a non-linear progression trajectory (Konerman et al., 2019). However, a benchmarked polygenic risk score which did not account for possible nonlinear effects, had a better prediction capacity for coronary artery disease than various ML techniques, such as penalized logistic regression, naïve Bayes, random forests, support vector machines, and gradient boosting when tested on an independent data set (Gola et al., 2020). This suggests that although overall ML strategies can improve the predictive capacity of individual or composite biomarkers commonly used in research or clinical practice, the added value of ML heavily depends on the quality and the relevance of the data fed into the model.

Machine Learning for Disease Subtype Identification and Therapy Selection

Personalised treatment is a fundamental aim of precision medicine, where individuals receive tailored therapy instead of the one-size-fits-all approach. The precision of the treatment is increasingly important in heterogeneous diseases, including autoimmune inflammatory diseases, where significant disease signature differences between patients can be overlooked by the same diagnosis. An effective way of delivering personalised treatment is by performing a more precise subpopulation identification based on their distinct pathogenetic signatures. Signatures can be extracted from genomes, metabolomics, immunophenotyping and other types of data. Supervised ML is an ideal tool, specialised in the identification of unique signatures, while clustering approaches from both supervised and unsupervised ML are designed for partitioning complex high dimensional data. An increasing number of studies have applied ML models to identify subgroups of patients and show promising results toward more personalised treatment (**Table 2**) (McKinney et al., 2010; Waljee et al., 2019; Mo et al., 2020; Rehberg et al., 2020).

SLE is a chronic ARD with no cure. Due to the heterogeneous nature of SLE, predicting treatment response of SLE patients remains challenging. Figgett and colleagues (Figgett et al., 2019) applied ML clustering approaches to perform SLE patient stratification using whole-blood RNA-sequencing data. Both unsupervised clustering (PCA, k-means clustering) and supervised clustering (partial least squares-discriminant analysis, PLS-DA) approaches were applied to the gene expression data from 161 SLE and 57 healthy samples. Unsupervised PCA provided an overall view of the gene expression data, which confirmed a higher heterogeneity in SLE compared with healthy controls. On the other hand, supervised PLS-DA maximised the difference between SLE and healthy controls with the help of labelled data, and selected top-weighted genes from the model. The SLE patients were then stratified into four clusters (C1–C4) with different gene expression signatures by k-means clustering. These identified clusters were supported by ML classifiers, where an 88% accuracy of model performance showed a clear divergence between these SLE subpopulations. From the enrichment analysis, C1 had the most similar gene expression architecture to healthy samples. Investigating the clinical manifestations of the clusters identified that flare activity was significantly elevated in C3 and C4; significantly more renal disorder and discoid rash in C4; significantly more serositis in C2. Moreover, using PLS-DA, genes related to disease flare were identified and used to discriminate between flare and non-flare patients, and enrichment analysis of the selected genes identified an increase in inflammatory signalling such as IL-6 and TNF-α, upregulated proliferation signalling, and haematological disturbances. This study improved the understanding of SLE heterogeneity and provides insight for potential personalised treatment in subpopulations of SLE patients.

In the recent study of primary Sjögren's syndrome (pSS) and SLE (Martin-Gutierrez et al., 2021), researchers applied supervised ML models to identify shared immunological characteristics between pSS and SLE. These two diseases share some clinical and laboratory features, despite differences in disease pathogenesis and overall clinical presentation, leading to a distinct diagnostic label (Pasoto et al., 2019). Immunophenotyping data comprising 29 immune cell subsets from 45 SS, 29 SLE, 14 patients with both conditions and 31 healthy controls was generated by flow cytometry. A range of analysis including supervised ML models (balanced random forest and sparse partial least squares discriminant analysis), univariate logistic regression and multiple t-tests were used to confirm the immunological similarity between pSS and SLE. Thus, all patient's data was then combined (n = 88) and stratified by k-means clustering into two groups with distinct immune profiles. The balanced random forest model identified a signature of eight T-cell subsets that differentiated between the two groups with high performance (AUC = 0.99). The 5 year clinical trajectory analysis identified differential damage scores and disease activity between the two groups. The study suggests the potential of differentiating pSS and SLE patients based on their immunological profile and could provide the opportunity for more accurate targeted treatments across diagnostic boundaries.

ML applications can be used to predict drug efficiency and provide precise treatment support for heterogeneous diseases. In a study of JIA (Mo et al., 2020), ML algorithms were employed to predict the efficiency of biological therapy (etanercept) in JIA patients using EMR data. A wide range of supervised ML approaches including extreme gradient boosting (XGBoost), random forest, gradient boosting decision tree (GBDT), extremely random trees and logistic regression were tested as potential predictive models. EMR data from 87 JIA patients receiving weekly etanercept treatment at the same dose (0.8 mg/kg) were used for model training. The efficacy of the etanercept treatment was assessed using a standard disease activity score validated in adults with RA (DAS44/ESR-3) (Ranganath et al., 2007; Consolaro et al., 2009) at baseline and 3 months after treatment, where a drop in DAS44 >0.6 was considered as a response to treatment. Feature selection was performed in each ML model. After optimisation, XGBoost outperformed the other models with an AUC 0.79 indicating a good predictive performance. Although an external validation was employed, this was small in number (only 14 patients) thereby limiting the reliability of the validation and the ability to apply the model in practice. Another study identified a limited contribution of genetic markers in addition to clinical parameters in predicting response to anti-TNF therapy in RA using a Gaussian process regression model which correctly classified patients' response in 78% cases (Guan et al., 2019). A recent ML application for personalised treatment response in RA investigated with success molecular signatures predictive of response to adalimumab and etanercept using differential gene expression in peripheral blood mononuclear cells (PBMCs), monocytes and $CD4^+$ T cells and methylation analysis in PBMCs (Tao et al., 2021). The random forest algorithms implemented to exploit the transcriptome signatures had an overall accuracy of 85.9 and 79% for response to adalimumab and etanercept and they have been validated in a partial dataset (a follow-up study).

Another study tried to predict anti-drug antibody development in MS patients treated with interferon β (IFNβ) (Waddington et al., 2020). More than one third of MS patients treated with IFNβ develop anti-drug antibodies, which significantly reduces drug efficacy (Bertolotto et al., 2002). Researchers quantified 228 serum metabolites and anti-drug antibody levels of 89 MS patients as part of the ABIRISK consortium (Hässler et al., 2020), at baseline (before treatment), 3 and 12 months after treatment initiation. Six supervised classification models (decision trees, random forest, kNN, SVM, logistic regression with and without interactions) were used to predict anti-drug antibody development (at month 12) and were validated by 10-fold cross validation. The decision tree model outperformed others with a F1 score of 0.788 and a classification accuracy of 0.854 using baseline metabolomics data as predictors. Similar models using serum metabolite levels 3 months after treatment showed better performance in predicting which patients will develop anti-drug antibodies at 12 months by logistic regression models (F1 = 0.88, accuracy = 0.863). The results from variable selection of the models and experimental validation, suggest that serum lipids might play an important role in anti-drug antibody development by changing the lipid composition of immune cell plasma membranes (lipid rafts). Together, this study demonstrates a potential methodology for efficient prediction of drug response using big data (omics and clinical data), which healthcare professionals can use to assess patients earlier for optimal treatment selection.

Machine Learning for Drug Development

Drug development is a complicated, costly, and time-consuming process, which depends on a large number of factors. The pipelines of drug development can be simply divided into two phases: the drug discovery phase and drug-testing phase (Réda et al., 2020). The drug discovery phase focuses on target identification, target validation and small molecule design, while drug-testing phase includes several preclinical and clinical trials. The complete timeline of drug discovery varies from 5 to 15 years (Réda et al., 2020) with more than 50% failure rate in the late clinical trial phase (Hwang et al., 2016). Due to the high-failure nature of drug development, developing automated approaches with high predictive performance is crucial. To date, numerous studies have investigated the application of ML in drug development (**Table 2**), aiming to improve the overall success rate by enhancing each step of the drug development process with the extensive use of big data (Vamathevan et al., 2019).

Target Identification and Validation

The first step of drug development is target identification which often heavily depends on the extensive study of disease mechanism. Understanding disease mechanisms can be time and labour intensive; common experimental techniques ranging from using immunoprecipitation assays to identify protein-protein interactions in biological samples to genome-wide CRISPR-Cas9 screens to knock down genes of interest. Modern high-throughput techniques generate abundant molecular and biological data, which makes it difficult to screen potential drug targets using conventional methods. To speed up the drug target selection process, numerous studies have developed automated in-silico approaches for drug target identification and validation.

A recent study by Zeng et al. (2020) developed a comprehensive deep learning approach called deepDTnet, which combines networks between drug, gene and disease data to identify novel targets for the existing drugs with great accuracy (AUC = 0.96). Retinoic-acid-receptor-related orphan receptor-gamma-t (ROR-γt) was selected from the deepDtnet approach, as having potential interaction with multiple drugs. An 18-drug screening panel of novel candidates selected from deepDTnet identified that Topotecan (a topoisomerase inhibitor) had an adequate ROR-γt inhibitory capacity (71.0% at 10 μM). Furthermore, this drug was able to ameliorate disease in an experimental mouse model of MS by targeting ROR-γt.

A Bayesian machine learning approach (BANDIT) developed by Madhukar and colleagues (Madhukar et al., 2019) integrated different data types such as treatment response, drug efficacy, molecular structure and adverse effect to predict unknown drug binding targets. The BANDIT model achieved an overall 90% accuracy on more than 2,000 small molecules. By applying the BANDIT approach on 14,000 small molecules with previously unknown targets, novel protein targets for 4,167 small molecules were confidently identified. Furthermore, by applying BANDIT to anti-cancer compounds in clinical development, Dopamine receptor D2 was identified and validated as a target and a compound targeting Dopamine receptor D2 is now undergoing clinical trials for cancer. Overall, BANDIT represents an efficient and accurate platform to accelerate drug discovery and direct clinical application. Together, these approaches overcome the limitation of using only known targets as input data, thus, can discover targets for orphan compounds.

Drug Repurposing

Drug repurposing is another powerful application aiming to discover, validate and apply existing approved drugs for new application. The process of drug repurposing is much conserved by renouncing the standard drug development pipeline approach and investigating similarities between various disease processes potentially targeted by the same therapeutic interventions, so that new effective treatments can be delivered faster to patients. This is a more cost-effective approach which led in recent years to the testing and licensing of similar classes of therapeutic agents across many immune-mediated chronic inflammatory diseases (March-Vila et al., 2017; Balasundaram et al., 2019; Gilvary et al., 2020; Martin-Gutierrez et al., 2021). In a recent study of computational drug repurposing, researchers developed a ML (Gradient Boosting model) approach, Creating A Translational Network for Indication Prediction (CATNIP) which can effectively connect similar drugs by solely analysing the biological and chemical data of the molecule without the knowledge of the current therapeutic disease applications of the drug (Gilvary et al., 2020). The CATNIP model was trained with 2,576 small molecules with a good model performance (AUC = 0.84). By performing CATNIP, a strong connection was identified between a kinase inhibitor drug (vandetanib) and diabetes, suggesting that vandetanib could be a potential treatment for type 2 diabetes.

TABLE 3 | Challenges in applying machine learning techniques in precision medicine for immune-mediated chronic inflammatory diseases.

· Robust models require sufficient high-quality data	Inadequate sample size in model development can lead to miss representation of the real population and model overfitting. Power calculations under universal guidance are essential during the study design process for ML studies
· External validation using independent datasets are an imperative step for predictive model implementation	Lack of external validation is markedly common in studies of autoimmune disease and raises several concerns including model overfitting, poor reproducibility, and generalisability. Online platforms with high-quality and well-defined datasets could enable data reuse which might help researchers with limited access to multiple cohorts to perform model validation
· Obstacles in model implementation in clinical practice	Limited interdisciplinary knowledge for translating model metrics to biologically relevant discoveries; lack of usable drugs for model stratified patients; and absence of significant improvement over the traditional approach. These can be improved by using standard practice guidance such as TRIPOD, which allow researchers to carefully assess their model for implementation
· Ethical concerns	Clinical predictive models rely on large amounts of personal healthcare data which raise the concern of private data leakage. AI/ML models can discriminate against groups based on ethnicity, gender or economic status due to reliance on biased "real world" data where minority groups maybe underrepresented

To date, many ML approaches have been applied to discovering effective drugs for acute respiratory syndrome coronavirus 2 (SARS-CoV-2). One of the studies selected the SARS-CoV-2 main protease (M^{pro}) as a potential drug target because it was highly conserved and encoded by a distinct gene. Due to the 96.08% similarity between SARS-CoV-2 and SARS-CoV (Xu et al., 2020), researchers hypothesised that inhibitors for SARS-CoV M^{pro} might be effective in blocking SARS-CoV-2 M^{pro} (Nguyen et al., 2020). By combing the mathematics analysis and deep learning models (MathDL), the binding affinity of 137 M^{pro}–inhibitors were predicted and ranked without any additional laboratory data. The model revealed that Gly143 was the most attractive residue in M^{pro} and 71 covalent bonding inhibitors interacting with the SARS-CoV-2 M^{pro} were identified. The study extended the current knowledge of the SARS-CoV-2 M^{pro} and provide important information for COVID-19 drug discovery.

Another study applied AI algorithms (BenevolentAI) to explore potential treatment options for COVID-19 using existing anti-cytokine therapies which enabled large-scale clinical trials to be rapidly conducted (Stebbing et al., 2020). Researchers aimed to identify existing drugs that could influence the COVID-19 infection progression by blocking the "cytokine storm" and reduce the associated inflammatory damage associated with a heightened immune response to the virus. Baricitinib is a (JAK)1/JAK2 inhibitor approved for RA treatment which was predicted to have an anti-viral (COVID-19) effect by the BenevolentAI algorithms. The following laboratory validation identified *in-vitro* and *in-vivo* evidence of a reduction in viral infectivity by baricitinib. In a pilot study, four COVID-19 patients were treated with baricitinib resulting in symptom improvement and viral load reduction, providing evidence for clinical benefit derived from ML-driven therapeutic target identification.

CHALLENGES

Despite the promise of ML research in the field of precision medicine, many challenges still need to be addressed to ensure the further development and acceptance of ML approaches (summarised in **Table 3**).

Data Quality

Being a data-driven approach, the performance of the ML model depends heavily on the quality of the data that it builds on. Data needs to have a sufficient sample size and quality in order to represent the target population in the clinical application. In general, a larger sample size is essential for the development of a more robust ML model, which allows accurate prediction for supporting clinical decisions. ML models trained by small sample sizes often suffer from the problem of "overfitting," where the model over relies on characteristics from the under-represented training data and loses the ability to effectively perform in practice. Similar to the multiple testing issue in conventional statistics, ML models with small sample size might cause false significant discoveries due to random variation under numerous repetitions. For example, one can generate 1,000 different splits of train/test data and evaluate performance. If the performance based on splits shows a great variance, this might indicate an "unstable" model. One way to improve model reliability due to small sample size is by reducing the model variance, as low variance algorithms are less influenced by the specificity of the training data. However, model variance reduction often results in an increase in model biased error, leading to a weakened predictive performance of models (Kohavi and Wolpert, 1996). Meanwhile, obtaining a larger sample size often requires more resources (time, funding, access to large patient populations and computer power etc.). One way to ensure the appropriateness of study design for the research outcome investigated is by having universal guidance of the adequate sample size required for the ML model training for researchers to follow. Studies have already attempted to develop tools to assist decision making in study design. For example, an r package "pmsampsize" was developed to calculate the minimum sample size for the predictive model development to avoid model overfitting, taking into account the number of participants, outcome events and predictive variables (Riley et al., 2019).

However, the use of a limited sample size can be sometimes inevitable due to the rare nature of certain diseases. To overcome the limitation of small sample size, more comprehensive procedures and careful considerations are necessary for generating reliable results. One example is juvenile-onset SLE (JSLE) – a rare ARD. In one study, researchers applied a ML model to stratify JSLE patients based on their immune profile (Robinson et al., 2020). Only 67 JSLE patients and 39 healthy controls with 28 immune cell predictors were included in the analysis. A random forest algorithm was selected as it was less likely to overfit the data due to an implanted bagging method and random feature selection in the model ensembled by a large number of decision trees (Tin Kam, 1995; Breiman, 2001). The results of this model were combined with additional analysis such as the sparse-PLS-DA and univariate logistic regression and were further validated by 10-fold cross-validation. Although the lack of an external validation dataset meant there was still risks for overfitting and not being able to extrapolate the results, the study shows the potential for applying a ML-based pipeline to other rare and heterogeneous immune-mediated inflammatory conditions (Choi and Ma, 2020).

Another challenge in the development of ML models is access to high quality and well-defined datasets, needed for algorithm training and evaluation. In recent years there has been a big push to make research data FAIR (Findable, Accessible, Interoperable and Reusable) (Wilkinson et al., 2016). Datasets generated in research studies should collect enough machine-readable metadata to allow for discovery and searches. Ideally, clear rules for data access and use should be available, as well as use of domain-specific ontologies to describe the data. There should also be enough information available describing how the acquisition of data was carried out, enabling re-use of data.

Reproducibility and External Validation in Machine Learning

Issues with multiple testing and p-hacking has contributed largely to the reproducibility "crisis" in science. The 2016 Nature survey pointed out that more that 70% of scientists have failed to reproduce other scientists reported results (Baker, 2016). P-hacking in traditional statistics usually means that tests are done on data in an exploratory manner, if something significant is found, a hypothesis is formed based on this finding, i.e., working backwards from data to find patterns and relationships. However, the statistical tests are only valid if the hypothesis is formed first. In ML, working backwards from data to reveal patterns is exactly what is done. In the case of ML, overfitting can be considered the analogy to p-hacking. Overfitting usually means that the ML model can perfectly reproduce training data, but fails on independent data. The way to handle this by data scientists is appropriate internal and external validation of models.

To achieve the highest model performance, many clinical studies tend to avoid data splitting for model development. Resampling methods such as bootstrapping and k-fold cross-validation are economical internal validation, therefore, they are often applied to prevent model overfitting. On the other hand, external validation using an independent cohort is not often performed, potentially due to limited access to similar cohorts, despite being the most straightforward way to evaluate the generalizability of the model. Less than 10% of autoimmune studies combine cross-validation with an independent test dataset for validating model performance (Stafford et al., 2020). However, external validation remains a crucial step for model implementation in real-world clinical practice and the absence of external validation will raise several concerns for the model integrity including bias of the model, lack of reproducibility and lack of model generalizability (Ho et al., 2020). One example is the publication of GWAS studies that are required to have at least two independent data sets for validation to assure a creditable result (Oetting et al., 2017). As external validation requires data from independent sources, access to publicly available online datasets from different studies has become a suitable solution to overcome the lack of independent validation cohorts (Riley et al., 2016). These online databases provide a great opportunity to improve the research quality of ML applications in immune-mediated inflammatory diseases that are often rare conditions associated with a limited number of datasets available. They provide options for researchers to validate their models on more relevant populations, as most current external validation studies use small local datasets simply because of the better accessibility.

Model Implementation in Clinical Practice

Transforming a well-performed model into an actual clinical application associated with improvement in patient outcomes can be challenging; the term "AI Chasm" describes the discrepancy between the model development and translation of models to real-world applications (Keane and Topol, 2018). The clinical impact of potentially promising ML models requires careful evaluation before considering implementation in clinical settings. For example, a wide range of performance metrics (accuracy, AUC, precision, sensitivity, specificity etc.) (see *Glossary*) are applied to represent the predictive efficacy of ML models in clinical studies. However, most of the metrics do not directly affirm the clinical applicability and can be difficult to evaluate with limited interdisciplinary knowledge (Saito and Rehmsmeier, 2015; Shah et al., 2019). Another common obstacle for the clinical translatability of ML data arrives where emerging ML studies that stratify patients with novel signatures suffer from the lack of effective drugs for the newly identified targets. Furthermore, the reported predictive model needs to provide clinically meaningful advantages over traditional approaches, such as significantly outperform the existing standard statistical approach in relevant fields (Shah et al., 2019). To help address these questions, standard practice guidance is necessary. Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD) guideline is an internationally accepted reporting guideline developed to improve the reliability and value of prediction models for diagnostic or prognostic purposes (Moons et al., 2015). TRIPOD-ML focuses on the standardised methodology of ML model development (Collins and Moons, 2019), which together with the interdisciplinary effort from trained experts in different clinical and technology areas of expertise, can ensure

that ML applications maximise their chance to translate into precision medicine approaches associated with patient benefit.

Ethical Concerns

The upsurge of ML applications in personalised medicine has raised potential ethical concerns regarding data privacy, as a wide range of big datasets including personal information from genetics data, demographic data and medication history are stored and used in various studies. Anonymisation is the most straightforward and common way for privacy protection of medical datasets by removing personal data for de-identification purposes. However, advanced re-identification techniques were developed and used to target the vulnerability of the anonymisation system by data mining companies, and data were then exploited by health insurance companies (Tanner, 2017). Thus, more rigorous data handling methods such as data decentralisation (storing data in separate locations) and federated machine learning (training algorithm across different decentralised local data) are necessary for institutes and companies dealing with large-scale personal data (Rieke et al., 2020). From patients and the general public's perspective, there is an innate scepticism related to the use of AI for clinical applications, especially with limited understanding about how ML and personal data are used in medical research. Face-to-face communication between specialists and patients is effective in conveying the scope of ML applications and addressing questions and concerns in terms of patient satisfaction (Mirzaei and Kashian, 2020). Public education events such as interactive Patient and Public Involvement and Engagement (PPIE) activities can inform patients about how AI and ML research can lead to better disease management and how data are handled within a secured framework. With a better understanding of ML approaches and how personal data are stored, used and protected, patients are more likely to engage with such research.

The phenomenon of ML algorithm-driven discriminating decisions has been well-observed in other areas of research using AI, such as racial discrimination in criminal charge facial recognition technology (Perkowitz, 2021) and gender discrimination in job recruitment algorithms (Yarger et al., 2019). Algorithm discrimination is not exempt in the clinical world. For example, an implemented algorithm in the US healthcare system for future health care needs prediction is heavily biased against black patients because of the lack of data on these patients (Obermeyer et al., 2019). This algorithm-intrinsic bias is inherited from existing inequality in society as black patients are generally less accessible to the healthcare system. Another study showed that the predicted hospital mortality of patients in critical care can vary by up to 20% according to their ethnic group (Chen et al., 2018). Many inflammatory diseases are independently associated with demographic variables such as age, sex and ethnicity. For example, autoimmune diseases are more frequent in the female population (Gleicher and Barad, 2007), which sometimes, for practical reasons, promotes research only within the most represented groups of patients, discriminating against the under-represented ones. Moreover, model development is highly data-driven with low tolerance to missing values in model training, which can also lead to potential bias by not capturing the real-life patient population of interest. For example, previous studies showed that vulnerable populations are less likely to attend the same clinic regularly due to limited access to healthcare, including diagnostic testing and medicines (Arpey et al., 2017; Gianfrancesco et al., 2018). Unintentionally excluding these incomplete datasets will lead to development of models that are less effective in populations with existing disadvantages. Thus, it is important for researchers and data scientists representing the diversity of the human condition to have opportunities to participate in the decision making and algorithm supervision process, assessment of the underlying biases associated with AI and ML and implementation of regulatory adjustments. This will avoid the development of discriminating decision-aiding algorithms.

The Future of Personalised Medicine

With such challenges evident at every possible step during the application of ML approaches, the ambition of personalised medicine to ensure that every individual receives an optimal treatment decision guided by their disease particularities and individual risk becomes uncertain. To warrant a future for ML applications in the clinical field, it is crucial to have universal procedure guidelines from data collection, data processing to model training, validation, and implementation (**Figure 2**). By ensuring the standardisation of ML applications, research study design can be optimised to facilitate granular and relevant data collection, as well as the use of an adequate sample size in relation to data multidimensionality to minimize the risk of significant data redundancy which can hamper the relevant patient identification (Plant and Barton, 2021). In addition, identification of reproducible biomarkers associated with response to therapy is one of the key requirements for personalized medicine approaches and we advocate for the use of truly independent data sets for validation. Although in theory, personalized medicine could be advanced by the use of ML algorithms for individual disease risk identification and prognostic, as well as therapy selection, its implementation in large health systems poses the ethical challenges of reconciling health risk inequalities with finite health care resources and standardised taxpayer or health insurance contributions (Rose, 2013). Future research should provide answers regarding the advantages of ML-driven personalised medicine strategies for long-term outcomes of patients in real-life.

CONCLUSION

The versatility of ML applications allows researchers to tackle divergent unmet clinical needs of immune-mediated inflammatory disease with the most effective tools (**Figure 1**). Predictive ML models with outstanding biomarker selection capability are crucial for developing diagnostic and prognostic approaches with high sensitivity and accuracy, which are particularly useful in the early stages of the disease, as well as for the long-term disease management and selection of therapies at every disease stage. Patient stratification by unsupervised models and advanced drug development strategies supported by deep learning providing a more personalised treatment selection is especially relevant for patients with immune-mediated chronic inflammatory diseases, because of

heterogeneity in clinical presentation, evolution and response to therapy. Despite several challenges which might impede some of the ML applications in clinical research and practice, the contribution of AI and ML techniques to personalised medicine for improved patient care is no doubt revolutionary.

AUTHOR CONTRIBUTIONS

CC, ECJ, and PD designed the study. JP performed the literature review and wrote the first draft of the manuscript. All authors reviewed the manuscript and approved the final version.

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GLOSSARY

Types of ML

Supervised Learning The type of ML algorithms which generates predictive models based on labelled training data. Two main types of supervised learning include classification and regression, capable of predicting category and continuous output, respectively.

Unsupervised Learning The type of ML algorithms which discovers underlying data structure based on unlabelled training data. Clustering is the main type of unsupervised learning.

Reinforcement Learning The type of ML algorithms which sequentially self-correct from either positive or negative environmental feedback to maximise the model function.

Deep Learning A subfield of machine learning that applies multiple layers of non-linear information processing for supervised or unsupervised feature extraction and transformation, using various neural network frameworks.

Main ML Model

Decision Tree A tree-like predictive model going from observation to prediction result by repeatedly splitting the data into data subset based on selected variables. There are two main types of decision tree (classification tree, regression tree) which serves different purpose (predict category result or continuous result).

Random Forest An ensemble classifier trained by a large number of unrelated decision trees. Bagging methods (or bootstrap aggregating) selects random samples from dataset when training each decision tree, which is applied in random forest models to improve model stability and accuracy.

Logistic Regression A supervised classifier used to predict the probability of a binary variable.

Naive Bayes A supervised classifier based on Bayes theorem. Naive Bayes models assume that the occurrence of a certain feature is independent of the occurrence of other features.

Support Vector Machine (SVM) A supervised learning model that builds a hyperplane in a high dimensional space for optimal separation between two classes, which can be used for classification and regression purposes.

K-Nearest Neighbours (kNN) A non-parametric classification algorithm which assigns the class of an unknown observation based on the class of a number (k) of similar observations in the feature space.

Artificial Neutral Network Algorithms that mimic the neural networks of the human brain. The artificial neuron (node) in a neural network processed the received signals and transmit to connected neurons. A neural network contains layers of interconnected nodes, where signals travel from the first layer (input layer), through the hidden layers eventually to the last layer (output layer).

Performance Metrics

Classification Accuracy (CA) The rate of correct classifications (number of correct predictions divided by the total number of predictions).

Confusion Matrix A 4x4 table showing the performance of a classification model. Rows represent the occurrences in the predicted class and columns represent the occurrences in the actual class.

Area Under Curve (AUC) An aggregated measure of performance of a binary classifier on all possible threshold values.

Precision Performance metrics for specific class.

Fraction of correctly predicted occurrences in a specific predicted class

True Positive/(True Positive + False Positive).

Recall (Sensitivity) Fraction of correctly predicted occurrences in a specific actual class

True Positive/(True Positive + False Negative).

F1 Score The harmonic mean of precision and recall.

$2 \times \text{Precision} \times \text{Recall}/(\text{Precision} + \text{Recall})$.

Gini Importance The average gain of purity (model improvement) by splits of a given variable. Replacing a more important variables usually cause a larger decrease in Gini-gain. ML Model such as random forest uses "mean decrease in Gini importance" to measure the variable importance in generating the model performance.

Other Terms

Overfitting Problem when a machine learning model fits too well to a particular dataset, causing it to lose generalization and predictive performance on other datasets.

Cross-Validation Model validation method designed to estimate the model performance when predicting new data which is not in the original model training, to avoid problems such as overfitting or selection bias. K-fold cross-validation randomly partitioning the data into "k" complementary subset. "k-1" portions will be used in model training and the remaining portion for validation, and process repeats until all data is used in model training and validation.

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REVIEW
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The Immunological Mechanisms and Immune-Based Biomarkers of Drug-Induced Liver Injury

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Drug-induced liver injury (DILI) has become one of the major challenges of drug safety all over the word. So far, about 1,100 commonly used drugs including the medications used regularly, herbal and/or dietary supplements, have been reported to induce liver injury. Moreover, DILI is the main cause of the interruption of new drugs development and drugs withdrawn from the pharmaceutical market. Acute DILI may evolve into chronic DILI or even worse, commonly lead to life-threatening acute liver failure in Western countries. It is generally considered to have a close relationship to genetic factors, environmental risk factors, and host immunity, through the drug itself or its metabolites, leading to a series of cellular events, such as haptenization and immune response activation. Despite many researches on DILI, the specific biomarkers about it are not applicable to clinical diagnosis, which still relies on the exclusion of other causes of liver disease in clinical practice as before. Additionally, circumstantial evidence has suggested that DILI is mediated by the immune system. Here, we review the underlying mechanisms of the immune response to DILI and provide guidance for the future development of biomarkers for the early detection, prediction, and diagnosis of DILI.

Keywords: drug-induced liver injury, mechanism, immune response, immune, biomarker

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INTRODUCTION

Drug-induced liver injury (DILI) is usually caused by regular medications, herbal and dietary supplements (HDS), manifested as liver damage caused by the drug itself or its metabolites. It has become the main reason for the interruption of drug development during clinical research as well as drugs withdrawal from the pharmaceutical market (Wysowski and Swartz, 2005). The incidence of DILI is 2.7–14 per 100,000 cases in Europe and the United States (Lei et al., 2015; Wang J. et al., 2015; Navarro et al., 2017), while that is about 23.8 per 100,000 cases in mainland China (Shen et al., 2019). In the United States, DILI is account for more than 50% of patients with acute liver failure (ALF) (European Association for the Study of the Liver, 2019). Acute DILI could evolve into chronic liver injury and reach hepatic failure, which requires liver transplantation, or even lead to death (Hayashi and Bjornsson, 2018). Therefore, DILI occurs throughout the life cycle of drug development and post-marketing scenarios, and it has become one of the major life-threatening public health events.

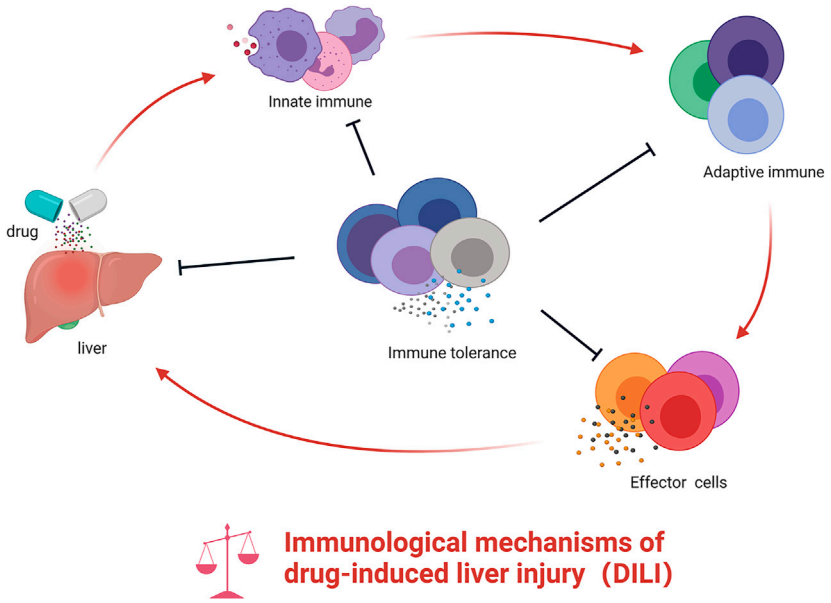

FIGURE 1 | The role of innate immunity and adaptive immunity in DILI. Drug or their reactive metabolites lead to cell stress, damage, or death, which release some molecules that recruit and activate innate immune cells prompting the release of pro-inflammatory cytokines. These mediators stimulate adaptive immune cells, ultimately resulting in the activation of T cells into effector cells and B cells into plasma cell–released antibodies. During the activation of innate and adaptive immunity, the host immune tolerance–related immune cells or cytokine may exert immunosuppressive effects. However, if the balance is broken, this will further aggravate the inflammatory response in the liver.

DILI is divided into intrinsic and idiosyncratic DILI (IDILI) according to the pathogenesis (Fontana, 2014). Intrinsic DILI, a predictable and rapid onset of liver injury in a dose-dependent manner, could be reproduced in animal models (Chalasani et al., 2014). In contrast, IDILI, an unexpected type of liver injury is mainly affected by a host of factors and accounts for 11% of ALF in the United States (Lucena et al., 2020). DILI is affected by multiple factors, including environmental exposures (drug dose, lipid solubility, drug interactions, and others) and genetic factors (drug metabolic enzymes, transporters, nuclear receptors, human leukocyte antigen (HLA), and others) (Björnsson, 2014; Chalasani and Björnsson, 2010). Age, gender, and pregnancy also influence the progression of liver injury caused by certain drugs (Dugan et al., 2011). At present, the clinical diagnosis of DILI is still based on biochemical detection of alanine aminotransferase (ALT) and aspartate transaminase (AST), and liver biopsy, still as the gold standard for confirmatory diagnosis. However, these methods not only lack specificity but also easily cause the host damage. Moreover, these cannot determine which drug should be responsible for liver injury given a the scenario of a combination of multiple drugs. Therefore, the development of mechanism-based specific biomarkers is significant for the prediction and diagnosis of DILI.

Existing studies have shown that mitochondrial dysfunction, oxidative stress, the imbalanced production and degradation of bile acid, and inflammatory responses are involved in the occurrence and development of DILI (Li et al., 2017; Kakisaka et al., 2018; Tu et al., 2019; Ejigu and Abay, 2020). However, these findings cannot fully elucidate the mechanism of DILI. The liver as an immune organ gathers several subsets of innate immune cells (e.g., neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, lymphoid cells, γδT cells, and others) and adaptive immune cells (such as T cells and B cells) (Ostapowicz et al., 2002; Adams et al., 2010) (**Figure 1**). Furthermore, the abnormalities of the above-mentioned immune cells and associated molecules affect the status of acute liver injury (Vega et al., 2017; García-Cortés et al., 2018; Stravitz and Lee, 2019). Recently, numerous clinical and experimental studies have found that immune responses are closely related to the development of DILI (Woolbright and Jaeschke, 2018). In particular, HLA alleles have been reported strongly associated with liver injury caused by a series of other drugs, e.g., flucloxacillin, clavulanic acid-amoxicillin, and *Polygonum multiflorum*, providing new insights for uncovering the mystery of DILI. In addition, some HDS-induced liver injuries have commonly observed antibodies or active T cells that support the immune system play a key role in the pathogenesis of liver injury (Manso et al., 2011; Wang et al., 2019). Here, we integrate the progress of the immune mechanisms of DILI and provide a reference for the prevention and treatment of DILI.

THE INNATE IMMUNE SYSTEM AND DRUG-INDUCED LIVER INJURY

The innate immune system is the first line of host defense, and its activation is much more rapid than the adaptive immune system (Patel et al., 2016). The innate immune system is a natural immune defense system, which is gradually formed in the

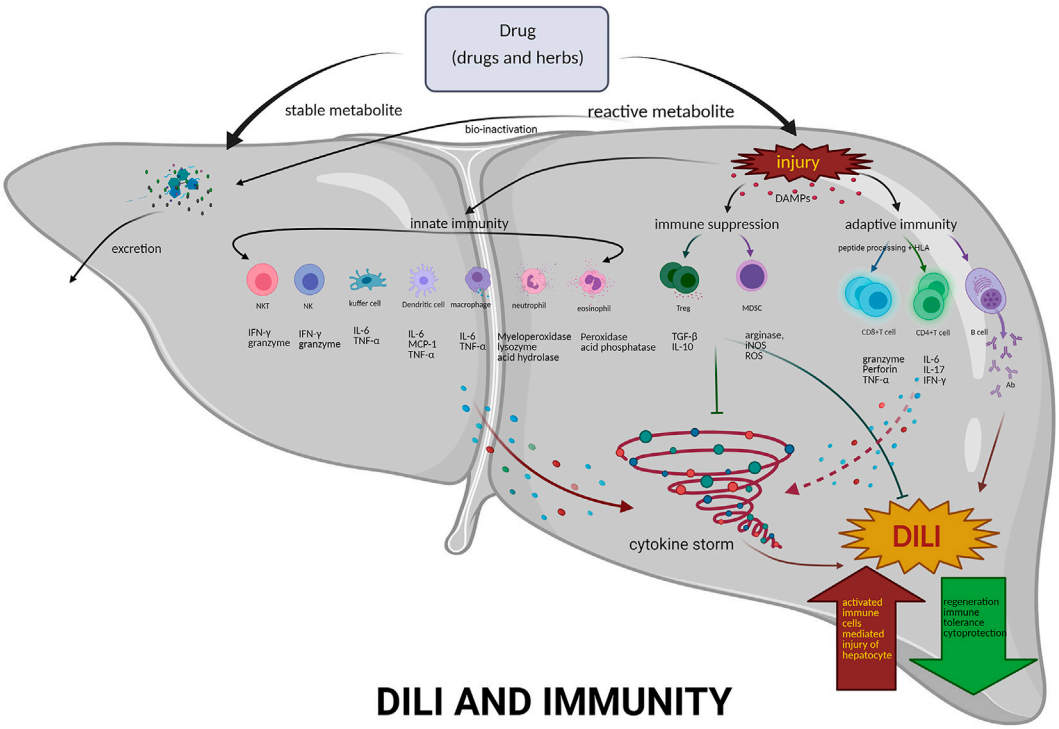

FIGURE 2 | Mechanisms of immunity in DILI. DILI is a disorder by drugs-induced liver damage, including pharmacological therapies, traditional medicines, and HDS. If the number of reactive metabolites produced exceeds the detoxification capacity, activated innate immune system–related cells, e.g., NKT, NK, dendritic cells, Kupffer cells, neutrophils and eosinophils, and the released various cytokines, form the cytokine storm. Furthermore, inducing adaptive immunity, activating T and B cells, producing effector T cells and antibodies, then liver damage all occur. However, Tregs may exert an immunosuppressive effect on APCs, effector T cells, and mast cells by the following mechanisms: coinhibitory receptors binding to cognate molecules on dendritic cells, secretion of inhibitory cytokines, e.g., IL-10 and TGF-β, metabolic disruption by depriving IL-2 binding and increasing adenosine binding to effector T cells, and contact-dependent cytolysis by granzyme B secretion. Abbreviations: NKT, natural killer T cells; NK, natural killer cells; Treg, regulatory T cells; MDSC, myeloid-derived suppressor cells; DAMPs, danger-associated molecular patterns; IFN-γ, interferon gamma; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; IL-10 interleukin 10; TGF-β, transforming growth factor-β1; IL-17, interleukin 17; Ab, antibody; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase.

long-term phylogenetic evolution of organisms, and is mainly composed of tissue barriers, innate immune cells, and molecules. Drugs or active metabolites could form new antigens to initiate innate immune responses and ultimately mediate immune liver damage. Damaged hepatocytes release related danger-associated molecular patterns (DAMPs) to activate innate immune cells, which secrete related cytokines and chemokines that recruit amounts of neutrophils and monocytes to the injured sites to clear necrotic cell debris. DAMPs include high-mobility group box protein 1 (HMGB1), S100 proteins, heat shock proteins, ATPs, and some others like mitochondria-derived DAMPs, reported to induce inflammasomes via binding Toll-like receptors 2 and 4 (Vénéreau et al., 2015). In turn, under certain conditions, the recruited immune cells could further aggravate liver injury by releasing a large number of pro-inflammatory factors to form a cytokine storm. Many other immune cells and the cytokines related to the innate immune response are involved in the occurrence of DILI, such as Kupffer cells (KCs), macrophages, type-1 innate lymphoid cells (ILC1s), NK cells, neutrophils, etc. These cells promote the inflammatory response by producing cytokines, chemokines, and reactive oxygen species (ROS) (Friedman et al., 2018), which in turn recruit immune cells to the site of injury to control the damage and initiate the adaptive immune response.

Kupffer Cells

KCs, as a specific type of macrophages residing in the liver, play a key role in immune-mediated liver injury (Dixon et al., 2013; Akai et al., 2016; Rose et al., 2016). Generally, KCs are divided into two types, M1 and M2. M1 KCs secrete pro-inflammatory cytokines, e.g., IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), which determine the inflammatory signaling pathway (Liu et al., 2015; Seo et al., 2018). However, M2 KCs have a weak antigen presentation ability and secrete potent immunosuppressive factors including IL-10 (Zeng et al., 2016). As an important subset of innate immunity in the liver, KCs could recognize danger signal molecules to scavenge dead liver cells (Li et al., 2017). During liver injury, KCs are complemented by infiltrating macrophages expressing distinct surface markers (Holt et al., 2008), up-regulating mitogen-activated protein kinase, and increasing the release of pro-inflammatory cytokines, which are important intermediate steps for immune-mediated liver injury (Zhang et al., 2019). Furthermore, KCs are also considered to exacerbate liver injury by oxygen free radicals

(Michael et al., 1999). Interestingly, in KC-depleted mice, the increased hepatotoxicity under acetaminophen (APAP) exposure suggested that KCs appear to have an effect of hepatoprotection (Ju et al., 2002), maybe due to the decreased expression of several hepatoregulatory cytokines like IL-10, an anti-inflammatory cytokine.

Dendritic Cells

In the liver, DCs as the main antigen-presenting cells (APCs) regularly divide into two subsets, classical DCs (cDCs) and plasmacytoid DCs (pDCs), and could initiate both innate and adaptive immune responses (Plitas et al., 2008; Steinman, 2008). cDCs as APCs express high levels of major histocompatibility complex (MHC)-II, while pDCs have a limited ability to present antigens due to their expressing relatively lower levels of MHC-II (Rahman and Aloman, 2013). Studies have shown that immaturity in liver DCs commonly mediates tolerance rather than immunogenicity as in the steady-state liver (Xia et al., 2008). DCs as hepatic immune system sentinels are a significant subset of the non-hepatocytes to alert the immune system during the presence of harmful pathogens. DCs also produce large quantities of cytokines, such as interferon-gamma (IFN-γ), promoting the activation of cytotoxic T cells. The mechanism of DILI is thought to be related to the interaction between APCs and T cells because of the damaged hepatocytes leading to the release of DAMPs, toxic components, and reactive metabolites, which aggravates liver injury and eventually results in liver failure (Grove and Aithal, 2015). Connolly et al. (2011) found that the immune-phenotype of DC in the liver significantly altered after APAP exposure, which expressed higher MHC-II and co-stimulatory molecules and increased the release of monocyte chemotactic protein-1, IL-6, and TNF-α. However, studies show that DCs may play a suppressed role in inflammatory response and reduce liver injury by releasing IL-10 (Krug et al., 2001). The trigger in the hepatic microenvironment that stimulates DC in diverse states of liver injury is undefined, but it may be the key component to understanding liver immunity.

Neutrophils

Neutrophils, as the most abundant type of innate immune cells, originate from the bone marrow and play a multifaceted role in host defense by phagocytosis, ROS, degranulation, and neutrophil extracellular trap for inflammatory responses (Jaeschke and Hasegawa, 2006; Döring et al., 2017). However, abnormal accumulation of neutrophils can lead to unexpected injury to host organs including DILI. Recently, more studies have defined that neutrophil activation commonly causes DILI, in which mitochondrial DNA originates from injured hepatocytes that could further activate neutrophils (You et al., 2006; Moles et al., 2014; Williams et al., 2014; Yuan et al., 2019). Liver injury increased expressions of chemokines, cytokines, and other immune molecules that could regulate neutrophil recruitment and activation, which cause cytotoxicity and hepatocyte death (Ramaiah and Jaeschke, 2007). In acute liver injury, activated neutrophils migrated into the hepatic parenchyma and recruited to the hepatic sinusoids can promote oxidative stress and necrosis, and further cause liver failure (Saiman and Friedman, 2012). It has been demonstrated that (Jaeschke, 2000; Fu et al., 2011) triptolide (TP) can cause stress response, lipid peroxidation, and hepatocyte necrosis, all of which can trigger neutrophil infiltration to further aggravate liver injury. Liu and Kaplowitz (2006) found that depletion of neutrophils could decrease serum ALT levels and centrilobular necrosis, in addition, ameliorate the progression and severity of APAP-induced liver injury. However, neutrophils caused host inflammatory response that could facilitate liver recovery by removing cell debris. Thus, the role of neutrophils remains controversial in DILI (Lawson et al., 2000).

Eosinophils

Eosinophils derive from myeloid cells, with high granulated shape and secreting cytokines and enzymes to kill pathogens or host cells (Kita, 2011). Eosinophilia has been often associated with DILI, including acetaminophen, diclofenac, carbamazepine, enalapril, and halothane (Dertinger et al., 1998; Pham et al., 2001; Aithal et al., 2004; Björnsson et al., 2007; da Silva et al., 2010). The report of severe eosinophilic hepatitis in patients treated with lamotrigine showed several features of hypersensitivity, including fever, rash, lymphadenopathy, eosinophilia, pneumonitis, increased liver enzyme levels, and the eosinophilic infiltration on biopsy examination (Fix et al., 2006). Drug reaction in liver injury with eosinophilia and systemic symptoms might be one type of DILI, with a different spectrum of culprit drugs. In a study of the mouse model (Proctor et al., 2013), eosinophils infiltrated the liver during early phase of halothane-induced liver injury by the secretion of pro-inflammatory cytokines, which increased proportionally to the hepatocellular damage (Kanda et al., 2009).

Natural Killer Cells

In humans, NK cells constitute 30–50% of hepatic lymphocytes (Nemeth et al., 2009; Qiao et al., 2018), and play a role in inspecting transformed or infected cells via the release of granzyme and perforin (Prager et al., 2019). In the liver, NK cells relate to physiological and pathophysiological processes, such as viral infections and other injuries, and participate in innate immune responses, cell-mediated cytotoxicity, as well as exocytosis of cytotoxic granules (Fasbender et al., 2016). These hepatic innate immune cells could participate in the pathogenesis of DILI. A double-stranded RNA viral mimetic that incurred the accumulation and activation of NK cells increased the halothane-induced hepatotoxicity in the mice model (Cheng et al., 2009). Indeed, NK cells can modulate DILI by IFN-γ production, resulting in hepatocytes cytotoxicity (Fasbender et al., 2020), and the cytotoxicity of NK cells are controlled by a sophisticated regulation of activating and inhibitory receptors (Godfrey et al., 2010; Bernardini et al., 2014). Evidence exist for the participation of NK cells in DILI, often involved in DNA damage, making histiocytes susceptible to NK cell lysis (Raulet et al., 2013). Studies also showed that the activation of NK cells have been claimed to be a key component in APAP-induced hepatotoxicity (Liu et al., 2004), and IFN-γ, a major source from hepatic NK cells, which has been shown to mediate immune cells infiltration, chemokine, and cytokine release and lead to

hepatocyte apoptosis (Ishida et al., 2002). IFN-γ has also influenced APAP and concanavalin A–induced liver injury in animal models (Tagawa et al., 1997). Administration of exogenous IFN-γ to patients with APAP caused elevated liver enzyme in the serum, implying that IFN-γ promotes liver injury in humans (Kenna et al., 1988). In a recent study, primary human hepatocytes exposed to 148 drugs of relevant concentrations in clinical by genome-wide analysis, found that several drugs, such as valproic acid, promethazine, ketoconazole, isoniazid, activated ligands for NK cell receptors like NKp30 ligand and NKG2D ligands and incurred hepatocyte killing by NK cells (Fasbender et al., 2020). The above studies support that NK cells activation can modulate DILI by IFN-γ production and interaction with hepatocytes.

Natural Killer T Cells

Natural killer T (NKT) cells, a unique subset of T cells, expressing both NK cell receptors and T cell receptors, are MHC class I–like molecules, CD1d-restricted and glycolipid antigen reactive (Wallace et al., 2009), and bridge innate and acquired immunity (Van Kaer et al., 2011), and are closely related to immune liver injury (Zheng et al., 2018). Distinct from conventional T lymphocytes, NKT cells preferentially taking the liver as their home, play a pathogenic role in various types of liver disease (Martin-Murphy et al., 2013; Schrumpf et al., 2015; Bandyopadhyay et al., 2016), secrete cytokines like IFN-γ, IL-4, and IL-17, and regulate the balance of pro- and anti-inflammatory responses in liver diseases (Wang and Yin, 2015; Bhattacharjee et al., 2017; Li and Hua, 2017). One case report on analyzing the hepatic and peripheral blood lymphocytes in two patients with drug-induced fulminant hepatic failure indicated that NKT cells might be involved in hepatic injury (Miyakawa et al., 2005). Studies have found that NKT cells are dominantly releasing IFN-γ and recruiting neutrophils and macrophages, leading to TP-induced liver injury (Wang et al., 2018). However, NKT cells are not only beneficial in APAP-mediated acute liver injury but also can limit inflammatory cytokine secretion (Kwon et al., 2014) because of type I and II cytokines secretion, making them both protective and harmful.

Type-1 Innate Lymphoid Cells

Innate lymphoid cells (ILCs) were originally found as liver-resident ILCs and characterized by the lack of receptors of B cells and T cells (Spits and Di Santo, 2011; Spits et al., 2013). ILCs sense pro-inflammatory cytokines at local tissue damage sites and immediately initiate innate immune responses in tissues (Nabekura et al., 2020). According to surface markers and secreted cytokines, ILCs are divided into three subsets: ILC1s, ILC2s, and ILC3s. In multiple tissues, ILCs can orchestrate homeostasis through multiple immune cell types (Eberl et al., 2015). Besides, activated ILC1s secrete IFN-γ and can upregulate Bcl-xL to inhibit acute liver injury (Nabekura et al., 2020). However, the immune regulation triggered by ILCs also contributes to the host protection against tissue repair, metabolism, and homeostasis (Klose and Artis, 2016; Ebbo et al., 2017; Colonna, 2018). At the same time, many studies have found that innate immune cells can repair acute liver injury under appropriate conditions (Hossain and Kubes, 2019), but the related mechanisms remain unclear.

THE ADAPTIVE IMMUNE SYSTEM AND DRUG-INDUCED LIVER INJURY

The adaptive immune response consists of humoral immunity mediated by B cells producing antibodies and of cellular immunity mediated by T cells (Shuai et al., 2016). In the liver, the adaptive immune system is indispensable in the pathophysiological processes of acute injuries (Gantner et al., 1995; Ke et al., 2013; Wang et al., 2015a). Many IDILI features with delayed onset and drug reactivation suggested that adaptive immune response could attack the liver and modulate individual susceptibility to liver injury (Chen et al., 2015). Some DILI patients upon drug discontinuation may develop persistent liver damage via releasing danger signals and activating innate and adaptive immune. Kim et al. (2015) found specific T cells infiltrated in amoxicillin–clavulanate–induced liver injury that seemed to be related to lymph toxin, resulting in the activation of IL-6 in the liver (Tumanov et al., 2009). However, due to the lack of valid animal models, in-depth studies of the immune mechanisms in DILI were virtually impossible.

T Cells

T cells differentiate and mature in the thymus and then migrate to the surrounding lymphoid tissue. According to the features and surface marks, T cells can be mainly divided into two subsets, $CD4^+$T and $CD8^+$T cells. But according to their function, these include cytotoxic T cells (CTLs), T helper cells (Th), regulatory/suppressor T cells (Treg), and delayed hypersensitive T cells. The liver histology of IDILI most commonly infiltrates some immune cells, often including $CD8^+$T cells (Mockenhaupt, 2014; Foureau et al., 2015). In addition, neoantigens by drug or its reactive metabolites in hepatocytes are presented by APCs and activate numerous $CD8^+$T cells in IDILI (Foureau et al., 2015). Simultaneously, study found that $CD8^+$T cells infiltrated in the liver of patients with flucloxacillin-DILI (Wuillemin et al., 2014). CTLs could kill target cells in different ways, such as by the activation of death receptors, granule exocytosis, and release of cytokines (Pinkoski et al., 2001; Metkar et al., 2003). Fulminant liver failure is probably caused by the infiltration of CTLs in drug adverse reactions (Amante et al., 2009). The infiltration of granzyme B^+CD3^+T cells was found around the apoptotic hepatocytes in patients with fulminant liver failure after vancomycin intake (Mennicke et al., 2009). Several MHC alleles associated with the susceptibility to IDILI present neoantigens to stimulate the activation and proliferation of T cells in IDILI (Daly and Day, 2012). The MHC-I molecules expressed on all nucleated cells present endogenous peptides for $CD8^+$T cells while the MHC-II molecules present exogenous peptides for $CD4^+$T cells (Jongsma et al., 2019). In addition, many HLA predict the risk of IDILI associated with MHC-I (Daly and Day, 2012), which also suggests that $CD8^+$T cell–mediated adaptive immune response causes most of IDILI. So, drug or drug metabolites modify proteins likely through the specific MHC molecule–initiated immune response.

B Cells

B lymphocytes stem from the bone marrow, and their size is slightly larger than that of T lymphocytes. Mature B cells emigrate from the peripheral blood and enter the spleen and lymph nodes. Following activation, B cells become plasma cells and produce antibodies participating in immune response. Recently, antidrug antibodies were observed in patients with amodiaquine, which suggests that the idiosyncratic drug reactions are immune mediated (Daly and Day, 2012). Most cases of severe isonicotinic acid hydrazide (INH)–induced liver injury were associated with antibodies against INH-modified proteins and native proteins, especially the cytochrome P450 (CYP) (Metush et al., 2014). The serum samples from patients with nomifensine were screened by immunoassay for IgE and IgG antibodies, and all patients had specific IgG antibodies (Wälti et al., 1983). In addition, patients with halothane-developed IDILI were found to have some antibodies against trifluoroacetylated proteins as well as autoantibodies against native proteins (Martin et al., 1993). The existence of antibodies can only indicate that DILI is immune-mediated, but there is no exact evidence to support that antibodies are the culprit of DILI. They could be explained as an immune response to liver damage or even to resolve the immune response.

THE IMMUNE TOLERANCE AND DRUG-INDUCED LIVER INJURY

The liver is an immune-privileged organ with complex immune responses and mainly provides protection through tolerating self or foreign antigens (Doherty and O'Farrelly, 2000). When the tolerance is impaired, the activated immune cells could release pro-inflammatory cytokines and chemokines to induce liver injury and hepatic inflammation, which determines the severity of liver injury (Wang et al., 2015b). The regulatory immune cells, immune checkpoint molecules, and other immune factors may participate in the balance of immune activation and tolerance. Numerous studies have shown that their abnormalities may also contribute to the pathogenesis of DILI.

Regulatory T Cells

Tregs maintain immune tolerance and homoeostasis of the immune system by inhibiting T cells activation and proliferation (Piccirillo and Shevach, 2001), blocking inflammatory cytokines release, and suppressing the immunoglobulins' production of B cells (Sakaguchi et al., 2010). $CD4^+CD25^+$ Tregs comprise approximately 5–10% of $CD4^+$T cells in human peripheral blood (Roncador et al., 2005). A recent study has shown that the diminishment of Tregs induces the loss of immune privilege in the liver and rapidly initiates an inflammatory response that causes liver damage (Lu et al., 2013). Due to Tregs being capable of suppressing immune cell-mediated hepatocytes damage (Wei et al., 2008; Stross et al., 2012), their depletion could result in the aggravation of ALF. Studies have found that the number of Tregs and the expression of foxp3 in the liver were decreased significantly after TP treatment and that adoptive transfer of Tregs could improve TP-induced liver injury, while depletion of Tregs decreased the levels of IL-10 and aggravated liver injury with higher levels of ALT and AST (Czaja, 2015; Wang et al., 2016). Indeed, Tregs were also reported to alleviate APAP-induced liver injury through IL-10 and transforming growth factor-β1 (TGF-β) (Wang et al., 2015a). Tregs negatively regulated liver NKTs likely in an IL-10–dependent manner (Hua et al., 2011), and might also depend on disrupting the balance of T cells (Gao et al., 2019).

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are heterogenous cells, which negatively regulate the immune system during infections, cancer, and other inflammatory conditions by direct cell–cell contact or secreting factors to suppress T cell responses (Gabrilovich and Nagaraj, 2009). MDSCs represent an intrinsic part of the myeloid–cell lineage comprised of the myeloid–cell progenitors and precursors of the myeloid cells. In pathological conditions, the activation of MDSCs could lead to the increased expression of immune suppressive factors such as inducible nitric oxide synthase, arginase, nitric oxide, and ROS. In addition, MDSCs not only have suppressive effects on adaptive immune responses but also show the regulation of innate immune responses via modulating the cytokine secretion from macrophages (Sinha et al., 2007). MDSCs infiltrate the liver and alleviate hepatotoxicity in experimental animal models of DILI (Liu et al., 2014). Furthermore, the depletion of hepatic MDSCs before halothane exposure could impair immune tolerance and aggravate liver injury (Chakraborty et al., 2015).

IMMUNE-BASED BIOMARKERS AND DRUG-INDUCED LIVER INJURY

Traditionally, in clinical practice, the diagnosis of DILI is always started by accurately tracing the history of drug administration and liver biochemical abnormalities by serum levels of ALT, AST, ALP, γ-GT (European Association for the Study of the Liver, 2019), and TBIL through history taking from patients. And then, according to the DILI diagnostic tool Roussel Uclaf Causality Assessment Method (RUCAM), making a probabilistic decision by a score card. Several causality assessment methods and the recently updated RUCAM have been developed based on scores (Danan and Teschke, 2015). The causality score is limited in the great challenge to differentiate DILI from other liver injuries. Even though the EASL (European Association for the Study of the Liver, 2019) DILI guidelines proposed definitions for DILI, traditional biomarkers have poor specificity in distinguishing DILI from other liver injuries. Thus, currently the diagnosis of DILI is mainly based on clinical criteria and the elimination of other causes (Fontana et al., 2010). The levels of enzymes in the liver also have an insufficient correlation to histological patterns of DILI (Devarbhavi, 2012). Although these biomarkers are useful in severe DILI diagnosis for their functions to reflect hepatic lesions, they have many limitations making them not ideal as biomarkers. Some newly proposed biomarkers are promising for the early detection of DILI but are not yet available for routine use in clinical practices, and still need confirmation on their specificity, validity, and sensitivity, particularly in comparison to that of traditional ones.

Human Leukocyte Antigen Polymorphisms

HLA, located on the human chromosome 6 short arm, is a gene complex composed of a series of tightly linked genes, encoding the MHC to regulate immunity. As aforementioned, a drug or its reactive metabolites such as haptens binding to proteins and then forming neoantigens, then presenting on specific HLA molecules, may initiate an inappropriate immune response that contributes to liver damage. The first successful genome-wide association study in DILI revealed flucloxacillin-induced liver injury with *HLA-B*57:01* (Daly et al., 2009). Subsequent studies found that whites carrying *HLA-B*35:02* were susceptible to minocycline-induced liver injury (Urban et al., 2017). Our research team (Li et al., 2019) had identified for the first time that *HLA-B*35:01* was a specific risk gene for *P. multiflorum*–induced liver injury (Rodriguez-Pena et al., 2006; Megherbi et al., 2009). Growing evidences have revealed that individuals carrying certain class I and II *HLA* alleles are at increased risk of DILI (Kindmark et al., 2008; Daly et al., 2009; Singer et al., 2010; Lucena et al., 2011; Spraggs et al., 2011; Nicoletti et al., 2016; Parham et al., 2016; Nicoletti et al., 2017; Urban et al., 2017; Kaliyaperumal et al., 2018; Nicoletti et al., 2019). Despite the strong association with *HLA*, the positive predictive value of *HLA* allele polymorphisms in drug-induced adverse reactions is limited (Rao et al., 2020). These results suggest that there are other factors, other than the *HLA* allele, that contribute to the progression of IDILI. Despite a lot of research in this field, the precise activation of the immune system and how it effects liver injury remain to be deeply defined, such as extremely limited sample sizes, lack of prospective studies, randomized controlled trials, and excessive confounding factors.

Antidrug Antibody

Idiosyncratic liver injury caused by drugs such as tienilic acid and halothane are associated with a variety of antibodies (Pohl et al., 1989; Bourdi et al., 1996; Robin et al., 1996), including antibodies against drug-modified proteins, anti-CYP antibodies, and other autoantibodies, as has been observed in DILI. The antibodies against amodiaquine (AQ) was detected in patients with liver injury induced by AQ, suggesting that AQ-induced idiosyncratic reactions represent an immune-mediated reaction against AQ-modified proteins (Christie et al., 1989). During therapy with nomifensine, specific IgE and IgG antibodies were identified (Wälti et al., 1983), and these findings may explain the immunological mechanisms of DILI and help in identifying patients at risk of serious adverse drug reactions.

Exosomes

Recently, exosomes as extracellular vesicles from various cells, secreted as membranous structures, have been studied as an important tool for intercellular communication. Exosomes are usually characterized as small membrane vesicles (diameter: 40–150 nm; density: 1.10–1.18 g/ml) (Kowal et al., 2016). Interestingly, some studies have considered the exosomes as potential biomarkers for the early evaluation, monitoring, and detection of DILI (Bala et al., 2012; Cho et al., 2017; Royo et al., 2017). When primary human hepatocytes were exposed to APAP, the level of miR-122 was increased in exosomes (Holman et al., 2016). In animal experiments, transcriptome profiling analysis of circulating messenger RNAs showed that the circulating liver-specific mRNAs in exosomes have the potential to be biomarkers for the diagnosis of DILI (Wetmore et al., 2010). A study reported that the mRNA in exosomes may have a cytotoxic effect in traditional Chinese medicine (TCM), which suggested that exosomal miRNAs can be used to deeply understand the mechanisms of TCM-induced liver injury (Zheng et al., 2018). *In vivo* experiments further demonstrate that exosomes significantly increased the number of Treg and decreased pro-inflammatory cytokine IL-2, which plays a key role in immunosuppressive effect (Zhao et al., 2021). Interestingly, human-derived stem cell exosomes could significantly improve the liver function, by decreasing hepatic apoptosis and modulating IL-1β, IL-6, and TNF-α levels in the mouse model of acute liver injury (Chen et al., 2017). However, the limitations of exosomes including the poor understanding and unclear mechanisms should be clarified.

Recently, the research on diagnostic and predictive biomarkers of DILI has aroused the enthusiasm of researchers, and many biomarkers, such as microRNAs, HMGB1, glutamate dehydrogenase (GLDH), and keratin-18, have been discovered (Antoine et al., 2009). Howell et al. (2018) recently published a review highlighting miRNA-122 being greatly sensitive and specific in predicting and monitoring DILI. However, a multicenter study tested the performance of several biomarkers and found that GLDH was more valuable than miR-122 in diagnosing DILI (Church et al., 2019). In the liver, a cytoskeleton protein was increased early before ALT, leading to a real damage of hepatocytes, which may be a prognostic marker of liver injury (Church and Watkins, 2017; Church et al., 2019; Uetrecht, 2019). To date, several genome-wide association studies have been conducted in DILI, however, the biomarkers that can accurately predict DILI have not yet emerged (Nicoletti et al., 2017; Urban et al., 2017; Barnhill et al., 2018); however, these have not yet predicted biomarkers for identifying DILI accurately.

CONCLUSION AND PROSPECTS

Clinicians mainly use exclusive diagnosis combed with causality assessment to improve DILI diagnostic, which is an important and arduous task for medical and health professionals. The pathological process of DILI is very complicated, and the specific mechanism has not been deeply elucidated mostly because of the lack of DILI animal models. The balance between immunity and tolerance is essential to liver function (**Figure 2**). Excessive inflammation may lead to liver injury, while insufficient immunity always allows for cancer or chronic infection. Dynamic interactions between the numerous subsets of immune cells in the liver are a key to DILI. Moreover, other factors involved in the immune response of DILI need to be clarified. A single biomarker is insufficient to accurately diagnose and predict the occurrence of DILI, thus more biomarkers need to be discovered and validated. Various new technologies of the fourth industrial revolution based on the combination of genomics, proteomics, and metabolomics will be developed and applied for the detection and diagnosis of DILI in the future.

AUTHOR CONTRIBUTIONS

DSOY conceived and designed the review. WHL, XCZ performed the review. DSOY reviewed and edited the manuscript. All authors read and approved the manuscript.

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CXCL12/SDF-1 in IgG4-Related Disease

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Background: SDF-1/CXCL12 is a chemokine with pleiotropic functions in hematopoietic stem cell niche homeostasis, germinal center architecture, B cell maturation, neoangiogenesis, and fibrosis. Recently, the CXCL12/CXCR4/CXCR7 axis was associated with cancer metastasis and autoimmune diseases. The IgG4-related disease (IgG4-RD) is a pathological condition characterized by IgG4+ plasma cells infiltrating fibrotic lesions. The aim of this research is to investigate the relevance of SDF-1/CXCL12 in IgG4-RD.

Materials and Methods: Peripheral blood samples were collected before therapy from a single-center cohort of 28 IgG4-RD patients, fulfilling the ACR-EULAR classification criteria. Clinical and serological data were obtained for each patient. In total, 14 healthy donors (NHS), 9 patients with pancreatic ductal adenocarcinoma (PDAC), and 9 with Sjogren syndrome (SSj) were recruited as controls and screened for circulating SDF-1/CXCL12 by ELISA. Moreover, paraffin-embedded pancreatic biopsies obtained from patients with IgG4-RD (n = 7), non-autoimmune pancreatitis (n = 3), PDAC (n = 5), and control tissues (n = 4) were analyzed to study the tissue expression and localization of SDF-1/CXCL12 and one of its receptors, CXCR4, and their potential relation with neutrophil extracellular traps (NETs).

Results: IgG4-RD patients had higher serum levels of SDF-1/CXCL12 than normal controls (p = 0.0137). Cytokine levels did not differ between the IgG4-RD autoimmune pancreatitis (AIP) and retroperitoneal fibrosis nor between the single- and multiple-organ involvement. No correlation was seen with the IgG4-RD Responder Index, IgG4 levels, white blood cells, or inflammatory markers in the serum. When compared to SSj, the IgG4-RD AIP subgroup presents higher amounts of serum SDF-1/CXCL12 (p = 0.0275), while no differences are seen in comparison with PDAC. The expression of SDF-1/CXCL12 in the tissue was significantly higher in the IgG4-RD tissue than the normal pancreas, and the tissue with the high SDF-1/CXCL12 expression is characterized by the overall inflammatory cell infiltration, fibrosis, and high level of NETs.

Conclusion: Modulating B cell development, neoangiogenesis and fibrosis, and SDF-1/CXCL12 may play a role in IgG4-RD. The higher levels observed in IgG4-RD, as compared

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to SSj, which closely mimics the disease, can be related to a different pattern of lesions, with prevalent fibrosis seen in IgG4-RD. Taken together, these findings suggest that drugs acting on the CXCL12/CXCR4/CXCR7 axis may affect IgG4-RD.

Keywords: IgG4-related disease, CXCL12, CXCR4, fibrosis, inflammation, NETs (neutrophil extracellular traps)

INTRODUCTION

IgG4-related disease (IgG4-RD) is a rare condition characterized by fibro-inflammatory lesions in one or many organs with peculiar histological features, such as tissue fibrosis with a storiform pattern, a diffuse lymphoplasmacytic infiltrate, obliterative phlebitis, mild to moderate eosinophil infiltrate, and abundance of IgG4+ plasma cells (Deshpande et al., 2012; Stone et al., 2012; Inoue et al., 2015).

Oligoclonal somatically hypermutated plasmablasts that produce IgG4 are detected in the peripheral blood (Mattoo et al., 2014; Doorenspleet et al., 2016). This population of B cells may be considered not only as a biomarker of the disease but also as a disease activity marker. In fact, plasmablasts are reduced during disease remission and reemerge during relapse (Wallace et al., 2015; Lin et al., 2017).

Little is known about B cell maturation and mutation in IgG4-RD, but tertiary lymphoid structures have been described in affected organs (Deshpande et al., 2012) and, as demonstrated in other disorders (Corsiero et al., 2016a), may represent sites of pathogenic B cell expansion.

The disorder can affect almost any organ: the pancreas, retroperitoneum, lymph nodes, salivary glands, and kidneys are the most frequently involved ones (Mahajan et al., 2014; Puxeddu et al., 2018a; Capecchi et al., 2021).

Diagnosis of IgG4-RD is based on a set of clinical, serological, and pathological criteria (Wallace et al., 2020), and the histological picture is critical for diagnosis.

The hallmark of IgG4-RD is represented by a typical pattern of fibrosis, rarely detected in other inflammatory disorders, with spindle cells (fibroblasts and myofibroblasts) radiating from a center (Deshpande et al., 2012). As in any fibrotic disorder, myofibroblasts, the main cells involved, exert their profibrotic activity deposing excessive extracellular matrix components, and the proportion of these cells decrease in advanced stages of the disease, characterized by a relatively acellular tissue.

Presently, few data are available on the circuits controlling the processes leading to tissue fibrosis in IgG4-RD. It has been suggested that a critical step in inducing the typical lesions is the release of profibrotic cytokines, but so far this hypothesis has only been partially investigated (Della-Torre et al., 2015; Puxeddu et al., 2018b; Capecchi et al., 2018).

Recent data draw attention to the role of SDF-1/CXCL12 as a mediator involved in recruiting circulating fibrocytes to the inflamed tissue in chronic periaortitis, a fibrotic disorder in the spectrum of IgG4-RD (Nicastro et al., 2019).

SDF-1/CXCL12 is a chemokine originally identified as a product of murine bone marrow stromal cells that interacts with two receptors, CXCR4 and CXCR7 (Karin, 2010). In embryonic life, this chemokine controls the proliferation and differentiation of immature progenitor cells (Nagasawa et al., 1996). In adults, SDF-1/CXCL12, constitutively expressed in bone marrow, the skin, the heart, and brain endothelium, regulates immature and maturing leukocyte trafficking to these tissues (Dar et al., 2006).

SDF-1/CXCL12 is also a potent chemoattractant for bone marrow eosinophils (Wong and Jelinek, 2013) and, in inflammatory processes, mediates leukocyte entry to inflammatory sites.

The pleiotropic effects of this chemokine suggest its possible involvement in multiple aspects of IgG4-RD.

In this study, we thus analyzed the serum levels of SDF-1/CXCL12 in IgG4-RD patients, focusing in particular on the subgroup with pancreatic involvement. The pancreatic expression of SDF-1/CXCL12 and its receptor CXCR4 was also studied in a subset of IgG4-RD patients with autoimmune pancreatitis (IgG4-RD AIP) and compared to what was observed in the pancreatic ductal adenocarcinoma (PDAC). In addition, the SDF-1/CXCL12 expression in the pancreatic tissue was evaluated in relation to the inflammatory infiltrate, particularly neutrophils, and neutrophil extracellular traps (NETs).

MATERIALS AND METHODS

Patients and Controls

In total, 28 patients fulfilling the criteria for the diagnosis of IgG4-RD were included in the study (Wallace et al., 2020). Here, 14 sex- and age-matched normal healthy subjects (NHS) were used as controls, and 8 patients affected by Sjogren's syndrome (SSj) (diagnosed according to ACR criteria) (Shiboski et al., 2017) and 9 affected by PDAC were also studied as disease controls. The IgG4-Related Disease Responder Index (IgG4-RD RI) was calculated as described by Carruthers et al. (2012). The study was approved by the local ethics committee (protocol 3661/2012), and patients gave their written informed consent.

Detection of Soluble Mediators

The levels of SDF-1/CXCL12 in the sera of IgG4-RD patients and controls were measured by ELISA (Human SDF-1/CXCL12 Quantikine, R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

Tissue Biopsies and H&E Staining

Paraffin-embedded pancreas biopsies from seven IgG4-RD patients, three not-autoimmune pancreatitis, five PDAC, and four normal tissues (adjacent to the tumor) were obtained from the Pathology Unit, Department of Surgical Pathology, University of Pisa. In order to confirm the original diagnosis, all the tissue sections were reviewed by an expert pathologist. All

the biopsies used in this study were fixed in a 10% buffered formalin solution, paraffin embedded, and cut at 3 μm. H&E staining was performed on all samples. Each sample was analyzed by an expert pathologist and scored from 0 to 3 for the level of fibrosis, follicles, lymphocytes, neutrophils, eosinophils, and plasma cells.

Immunofluorescence Staining for CXCR4, SDF-1/CXCL12, and NETs

Deparaffinized pancreatic biopsies underwent antigen retrieval at 95°C with a low pH buffer solution followed by block of not-specific binding. The sections were then incubated with the proper combination of primary antibodies; for CXCR4 and SDF-1/CXCL12, the slides were incubated overnight at 4°C with a goat anti-mouse IgG1 antibody specific for SDF-1/CXCL12 (R&D), and after PBS washing, the sections were incubated for 1 h at RT with a goat anti-mouse IgG2b antibody specific for CXCR4 (R&D). For detection of NETs, the slides were incubated for 1 h at RT with a goat polyclonal anti-rabbit antibody specific for MPO (Dako) and a goat anti-chicken antibody specific for H2B (Abcam).

After washing, the sections were incubated with the appropriate combination of fluorochrome-conjugated secondary antibodies. Alexa 488–conjugated goat anti-mouse IgG1 and Alexa 555–conjugated goat anti-mouse IgG2b (Invitrogen) were used for SDF-1/CXCL12 and CXCR4, respectively. Alexa 488–conjugated goat anti-rabbit and Alexa 555–conjugated goat anti-chicken (Invitrogen) were used for MPO and H2B, respectively.

Cell nuclei were counterstained with DAPI. Negative controls included omission of the primary antibody followed by the proper secondary antibody.

All the sections were visualized using an Olympus BX-41 microscope. Stained cells were counted in five 40X microscopic fields per section.

Analysis of Immunofluorescence Staining

The presence of SDF-1/CXCL12 and its receptor CXCR4 was evaluated by a semi-quantitative score. In detail, in each tissue slide, both CXCR4 and SDF-1/CXCL12 were scored from 0 (no cells) to 3 (high number of cells). A median value was obtained for the IgG4-RD sample tissues and control groups (not-autoimmune pancreatitis, PDAC, and normal tissue). Both SDF-1/CXCL12 and CXCR4 expression levels were evaluated in relation to the level of fibrosis and the presence of follicles, lymphocytes, plasma cells, eosinophils, and neutrophils, previously evaluated by H&E staining. Finally, in the same tissue, both CXCR4 and SDF-1/CXCL12 expressions were correlated with NETs (evaluated using immunofluorescence staining by the co-expression of MPO and H2B in five 40X microscopic fields per section). The levels of SDF-1/CXCL12 were considered low with a score of 0 or 1 and high with a score of 2 or 3.

Statistical Analysis

A non-parametric Mann–Whitney *U*-test was used to compare the patients' group and the normal subjects. $p < 0.05$ was considered statistically significant.

TABLE 1 | Demographic and serological characteristics of IgG4-RD patients.

	IgG4-RD patients (*n* = 28)
Age (years) (mean and range)	62 (39-81)
Sex (M/F)	17/11
Disease duration (months) (mean and range)	23 (0-413)
IgG (mg/dl) median (IQR)	1342 (1111–1833)
IgG4 (mg/dl) median (IQR)	210 (99.5–218)
IgG4/IgG (%) median (IQR)	17.57 (11.78–23.16)
IgE (mg/dl) median (IQR)	57 (15.5–98)
ESR (mm/h) median (IQR)	25 (10–70)
CRP (mg/L) median (IQR)	0.4 (0.16–1.13)
Eosinophils (n°/μl) median (IQR)	200 (87.5–282.5)
IgG4-RD RI median (IQR)	7.5 (5–10)

IQR, interquartile range; ESR, *erythrocyte sedimentation rate;* CRP, *C-reactive protein;* IgG4-RD RI, *IgG4-related disease Responder Index.*

RESULTS

Patient Cohort

The clinical and serological features of the IgG4-RD patients are summarized in **Table 1**.

In total, 13 patients had a single-organ involvement, while 8 patients had a two-organ involvement, and 7 had multi-organ diseases (three or more organs affected). The most frequent organs involved were pancreas ($n = 13$); retroperitoneum ($n = 9$); lymph nodes ($n = 8$); salivary glands ($n = 7$); orbits and lacrimal glands ($n = 4$); the kidney, liver, and thyroid ($n = 3$); and lungs, large vessels, and the pituitary gland ($n = 2$). Skin involvement was observed only in one patient. The median of the IgG4-RD Responder Index was 7.5, indicating an active disease in most of the patients. The median of circulating eosinophils was 200/μl (87.5–282.5 IQR) with a value > 700/μl in 2 out of 28 patients. Three patients were previously treated with immunosuppressant (rituximab or cyclophosphamide), while eight patients were under treatment with low-dose steroids (mean dose 8 mg/die prednisone equivalent, SD ± 11).

Increased SDF-1/CXCL12 Serum Levels in IgG4-RD

SDF-1/CXCL12 serum levels were significantly higher in IgG4-RD patients compared to NHS ($p = 0.0142$) (**Figure 1A**). The subgroup of IgG4-RD AIP was compared with SSj and PDAC patients. SDF-1/CXCL12 levels were higher in IgG4-RD AIP than in SSj patients but comparable to those observed in PDAC (**Figure 1B**).

In IgG4-RD patients, the levels of SDF-1/CXCL12 were not related to inflammatory markers (erythrocyte sedimentation rate or C-reactive protein or disease activity evaluated by the Responder Index (data not shown)). Although in the literature it has been demonstrated that SDF-1/CXCL12 is an eosinophil chemoattractant (Nagase et al., 2001), we did not found any correlation between the serum level of this cytokine and the number of circulating eosinophils.

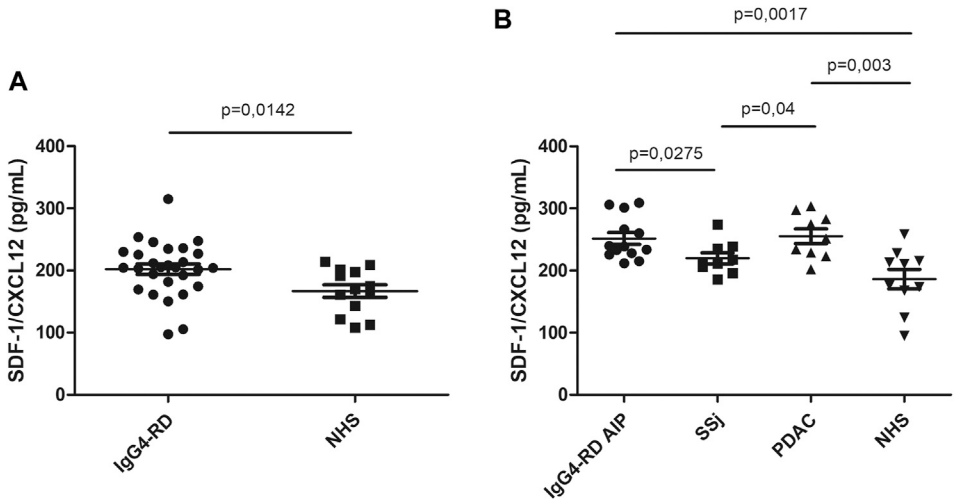

FIGURE 1 | SDF-1/CXCL12 serum level in IgG4-RD patients and healthy controls (NHS) **(A)**. SDF-1/CXCL12 serum level in IgG4-RD autoimmune pancreatitis (AIP), Sjogren syndrome (SSj), pancreatic adenocarcinoma (PDAC), and healthy controls (NHS) **(B)**.

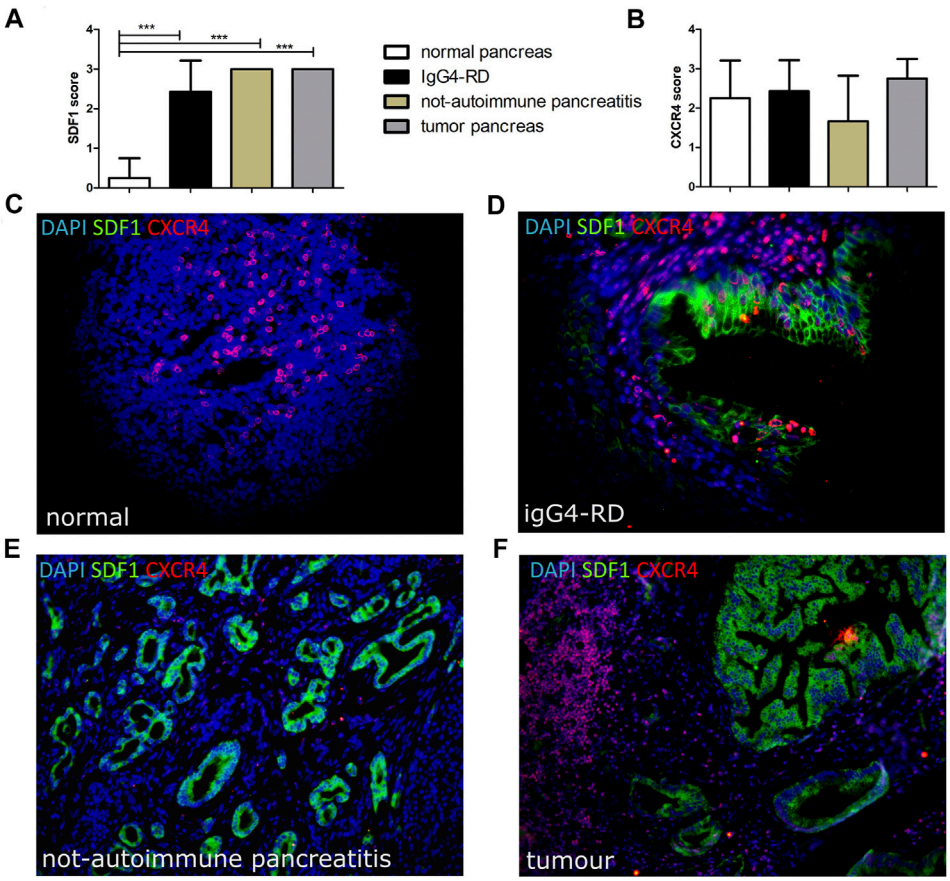

FIGURE 2 | SDF-1/CXCL12 **(A)** and CXCR4 **(B)** score in pancreatic tissues from normal, IgG4-RD, not-autoimmune pancreatitis, and tumor pancreas. A representative image of a double staining SDF-1/CXCR4 in normal pancreas **(C)**, IgG4-RD pancreas **(D)**, not-autoimmune pancreatitis **(E)**, and tumor pancreas **(F)** is shown. *** = $p < 0.001$.

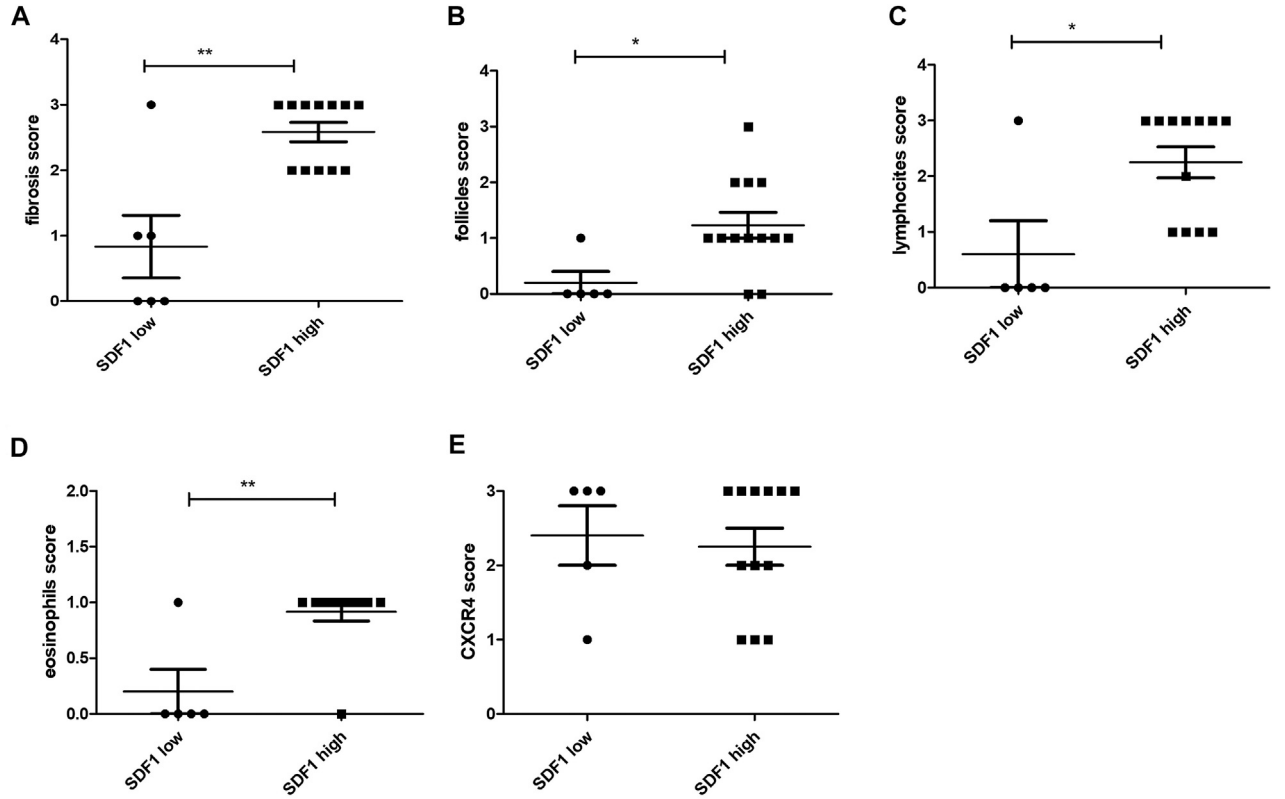

FIGURE 3 | SDF-1/CXCL12 expression in the pancreas tissue related to the fibrosis score **(A)**, the follicle score **(B)**, the lymphocyte score **(C)**, the eosinophil score **(D)**, and the CXCR4 score **(E)**. * = $p < 0.05$; ** = $p < 0.01$.

Increased SDF-1/CXCL12 Expression in IgG4-RD Tissue

The SDF-1/CXCL12 expression was significantly higher in IgG4-RD with respect to normal pancreas tissue, comparable to those found in different disease groups including PDAC and in not-autoimmune pancreatitis (**Figure 2A**). Conversely, we did not find any significant difference in the CXCR4 level of expression and localization among the different disease groups (**Figure 2B**). SDF-1/CXCL12 was mainly expressed in ducts and acini whereas CXCR4 was very rarely expressed in SDF-1/CXCL12-positive cells (**Figures 2C–F**).

SDF-1/CXCL12 Expression and the Overall Inflammatory Cell Infiltration and Fibrosis

In the pancreatic tissues with high expression of SDF-1/CXCL12, significantly higher levels of fibrosis (**Figure 3A**), follicles (**Figure 3B**), lymphocytes (**Figure 3C**), and eosinophils (**Figure 3D**) were also found. On the contrary, no differences between high and low expression of SDF-1/CXCL12 were observed stratifying for the CXCR4 expression (**Figure 3E**). Similarly, no differences between the CXCR4 expression and the level of fibrosis, the number of follicles, lymphocytes, neutrophils, eosinophils, or plasma cells were detected (data not shown).

An Aberrant Level of NETs Is Characteristic of Tissue Expressing High Levels of SDF-1/CXCL12

As reported in **Figures 4A-C**, NETs were more frequent in pancreatic tissues from IgG4-RD and PDAC compared to the control. Semi-quantitative analysis of the NET expression revealed a significantly increased level of NETs in IgG4-RD and PDAC pancreatic tissues compared to normal control ($p < 0.05$ and $p < 0.01$, respectively) (**Figure 4D**). By analyzing the data from all the pancreatic tissue biopsies, we observed in the pancreatic tissues with high expression of SDF-1/CXCL12 a significantly higher level of neutrophils (**Figure 4E**) and NETs (**Figure 4F**) than in the tissues with low SDF-1/CXCL12 expression ($p < 0.05$ and $p < 0.01$, respectively).

DISCUSSION

In this study, we analyzed the serum levels of SDF-1/CXCL12 in IgG4-RD patients and the pancreatic expression of the

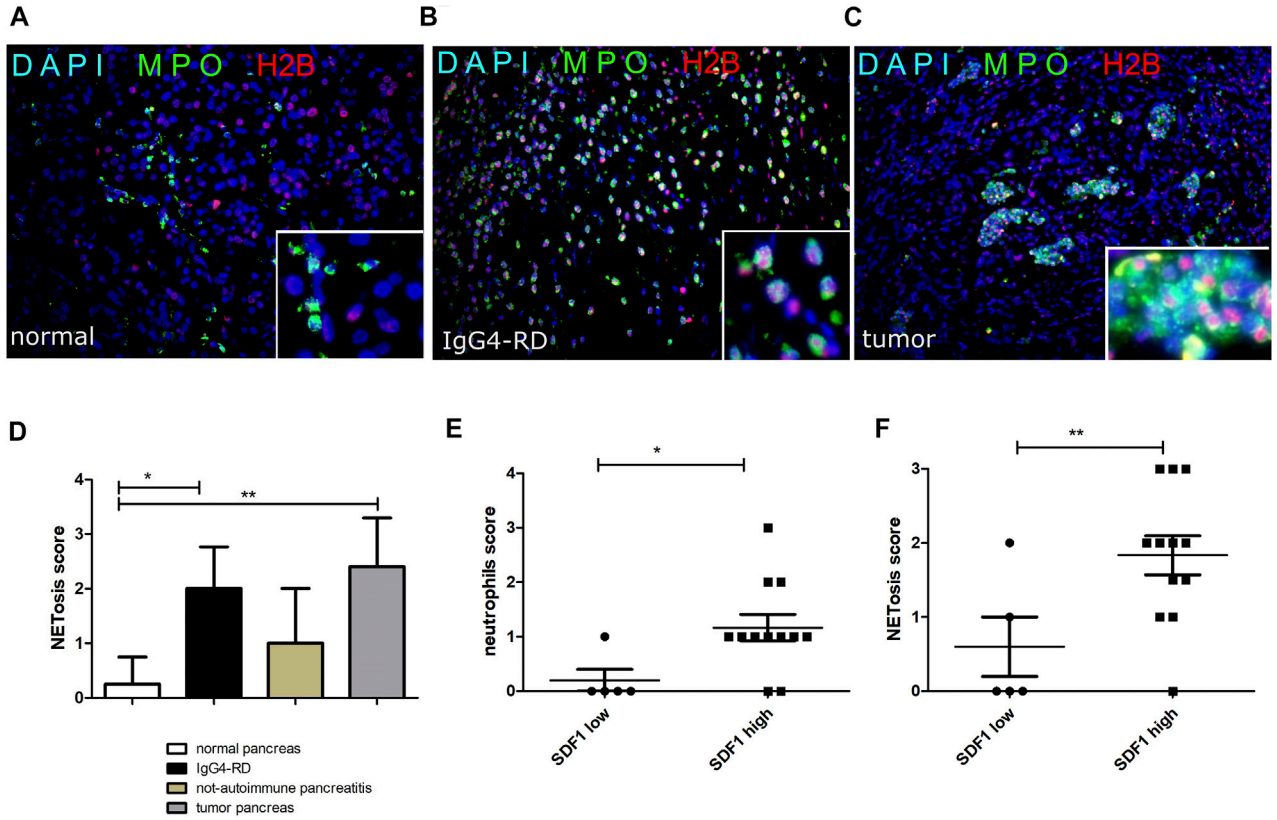

FIGURE 4 | NETs (double H2B and MPO staining) in normal pancreatic tissue **(A)**, IgG4-RD pancreas **(B)**, and tumor pancreas **(C)**. A semi-quantitative analysis of NETosis in the different groups is reported **(D)**. The neutrophil **(E)** and NETosis **(F)** score in pancreas reflects the SDF-1/CXCL12 expression in pancreatic tissue. $* = p < 0.05$; $** = p < 0.01$.

chemokine in the subgroup of IgG4-RD with autoimmune pancreatitis.

The results indicate that chemokine is overexpressed in the sera of IgG4-RD at levels comparable to what was observed in PDAC patients. SDF-1/CXCL12 serum levels are not correlated with the disease activity index or with acute phase reactants and, thus, cannot be considered a biomarker of disease exacerbations. Patients affected by chronic periaortitis, a fibro-inflammatory condition in the spectrum of IgG4-RD, show a similar increase in SDF-1/CXCL12 serum levels (Puxeddu et al., 2018a). Only a subset of chronic periaortitis patients, however, can be diagnosed as IgG4-RD. According to our findings, SDF-1/CXCL12 is elevated in all IgG4-RD patients, especially in those with pancreatic involvement. Under this respect, the overexpression of the chemokine in the pancreas from AIP, not-autoimmune pancreatitis, and PDAC is of interest as compared to normal tissue. In all these conditions, the SDF-1/CXCL12 expression is correlated with overall fibrosis, suggesting a role of this chemokine in fibrotic tissue remodeling in all these disorders. SDF-1/CXCL12, locally produced mainly by fibroblasts and hepatic stellate cells, exerts profibrotic activity by multiple mechanisms. Activation and proliferation of fibroblasts with increased production of extractable matrix components have been described as the effect of the chemokine (Jackson et al., 2017). A second mechanism is tissue recruitment of circulating fibrocytes and mesenchymal progenitor cells ($CD45^+$, collagen-1+) that migrate to sites of injury and play an important role in disorders characterized by excessive collagen deposition (Herzog and Bucala, 2010). Fibrocytes produce a number of extracellular matrix proteins, including collagen-1, collagen-3, and fibronectin (Ashley et al., 2017). In a mouse model of lung fibrosis, it has been shown that human fibrocytes migrate in response to SDF-1/CXCL12 and localize to lungs if injected in bleomycin-treated animals (Phillips et al., 2004). An increase in the number of collagen-positive fibrocytes was detected in chronic periaortitis patients, both in peripheral blood and in the affected tissue (Nicastro et al., 2019). Similarly, in IgG4-RD patients, SDF-1/CXCL12 may recruit fibrocytes to affected organs, driving tissue fibrosis.

In PDAC, overexpression of SDF-1/CXCL12 has often been reported, at least in part, mediated by the production of the hypoxic inducible factor (HIF) in the hypoxic tumor environment. The profibrotic activity of the chemokine supports tumor growth, protecting cancer cells from the host's immune system and creating niches for metastatic growth (Righetti et al., 2019).

In the tissue sections we studied, the SDF-1/CXCL12 expression reflects the number of lymphoid follicles, and eosinophil and neutrophil infiltrate.

SDF-1/CXCL12 plays an important role in B cell homing and differentiation in germinal centers. Ectopic germinal centers have been described in salivary glands of IgG4-RD patients (Maehara et al., 2012), and the role of SDF-1/CXCL12 in the formation of these ectopic lymphoid structures are well established (Corsiero et al., 2012).

Thus, it is not surprising that in different disorders, the SDF-1/CXCL12 expression is related to the number of lymphoid follicles.

The relation with eosinophil and neutrophil infiltrates suggests the role of SDF-1/CXCL12 in mediating the migration of these cells into pancreatic tissue. SDF-1/CXCL12 is by itself a weak neutrophil chemoattractant but synergizing with other chemokines like IL-8 may increase neutrophil infiltration (Janssens et al., 2018).

SDF-1/CXCL12 signaling is mediated by the interaction with CXCR4, a G-coupled receptor that is activated also by ubiquitin, and by the binding of a macrophage migration inhibitory factor. CXCR4 is diffusely expressed in the pancreas, indicating that the chemokine may exert its activities in the tissue through receptor binding. No differences in CXCR4 distribution or density were detected in different diseases we studied; thus, chemokine and the receptor expression are not parallel and in fact has been reported that the CXCR4/SDF-1 system is regulated at the level of the receptor expression (Nagase et al., 2001). However, these data do not allow drawing firm conclusions on the functional activity of SDF-1/CXCL12 in the pancreas.

In fact, splice variants of SDF-1/CXCL12 give rise to different isoforms with a tissue-specific pattern of expression: CXCL12δ, CXCL12ε, and CXCL12ϕ are most abundantly expressed in the pancreas. The isoforms share the first 67 amino acids but differ in length and in signaling efficiency. Only splice variant-specific antibodies may shed light on the pattern of the isoform expression in the tissue. Moreover, the biological activity is regulated by posttranslational modification of the chemokine, such as citrullination and tyrosine nitration (Struyf et al., 2009; Janssens et al., 2016).

SDF-1/CXCL12 may also contribute to organ damage, mediating neutrophil infiltration of the pancreas.

In IgG4-RD and PDAC, neutrophils infiltrating the pancreas frequently undergo NETosis, a form of programmed death that represents a critical defense mechanism in innate immunity but also a potential mechanism of tissue damage. By the extrusion of chromatin fibers coated with granule enzymes and other cytoplasmic constituents, neutrophils entrap the microorganisms too big to be phagocytosed (Brinkmann et al., 2004). NETs, however, can be directly harmful to the surrounding cells, can lead to the production of ROS and other inflammatory mediators, and may also represent a molecular platform for autoantibody induction (Corsiero et al., 2016b).

In PDAC, IL-17 mediates neutrophil recruitment and induces NETosis. NETs enhance migration and activate pancreatic stellate cells that form dense stroma and enable tumor proliferation (Miller-Ocuin et al., 2019).

One of the NET constituents is the antimicrobial peptide cathelicidin, which forms complexes with RNA; complexed RNA is protected from degradation and transported into the endosomal compartment of dendritic cells where it stimulates the production of IFN alpha (Ganguly et al., 2009). Moreover, activation of dendritic cells is triggered by RNA-cathelicidin complexes. A dense plasmacytoid dendritic cell infiltrate characterizes the affected pancreas in AIP. As reported by Arai et al., in IgG4-RD patients, NET-stimulated dendritic cells release IFN alpha and induce the production of IgG4 in cocultured B cells (Arai et al., 2015). Thus, in IgG4-RD and PDAC, SDF-1/CXCL12 may affect inflammatory cell migration and B cell homing in the pancreas. The stimuli responsible for NET formation may differ in the two disorders, and the contribution of NETosis to disease progression in PDAC and IgG4 production should be further explored.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics committee (protocol 3661/2012). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IP, PM, and RC contributed to the conception and design of the study. IP, RC, and CC performed all the experiments and analyzed the results and the clinical and serological data. CC, FP, and RC performed the statistical analysis. IP, PM, RC, and CC wrote the first draft of the manuscript. RC, AT, LM, UB, DC, and AC recruited the patients, provided the biological samples, and collected all the clinical data. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Precision Medicine in Graves' Disease and Ophthalmopathy

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Graves' disease (GD) is a condition caused by an autoimmune process involving the thyroid gland, whose main outcome is hyperthyroidism. TSAb start the autoimmune process stimulating the overproduction of thyroid hormones. In addition, TSAb can stimulate TSH-R expressed in fibroblasts and orbital pre-adipocytes leading to the manifestation of Graves' ophtalmopathy (GO). Also, autoantibodies directed against IGF-1R have an important role in immune-pathogenesis of GO. Fundamental is the role played by cytokines (IFN-γ, TNF-α, Il-6), and Th1 chemokines in the immune-pathogenesis of both disorders, particularly in the active phase. Novel discoveries in the field led to the investigation of promising therapies, such as immune-therapies towards specific antigens (for example against TSH-R), aiming in restoring the immune tolerance versus the immune dominant epitopes associated with autoimmunity in GD. Moreover, Etanercept (that blocks the TNF-mediated inflammatory responses), TCZ (that acts against the IL-6 receptor), and RTX (that acts against CD20) have proven to be useful and safe therapeutic options in refractory GO treatment. Furthermore, teprotumumab (a human monoclonal anti-IGF-1R blocking antibody), have been revealed effective in the treatment of patients with moderate-severe GO and it is now approved for GO therapy in United States. Molecules able to act as antagonists of CXCR3, or to block CXCL10, are also under study. More extensive researches are needed to deepen out these drugs as well as to identify new targeted and effective therapies, that will permit a more precise identification of GD, or GO, patients able to respond to specific targeted therapies.

Keywords: Graves' disease, Graves' ophthalmology, thyroid eye disease, teprotumumab, tocilizumab, rituximab, chemokine, cytokines

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INTRODUCTION

The immune system has the important role to protect our body from foreign or inner attacks, but unfortunately this delicate mechanism can be broken and our immune system can attack the self-antigens leading to the appearance of autoimmune disorders. Several factors can contribute to this breakdown, such as environmental, genetics, immunological, hormonal conditions, being part of the "mosaic of autoimmunity" (Shoenfeld et al., 2019). Nowadays autoimmune disorders are largely widespread and in rising growth with women representing the mostly affected gender; moreover,

autoimmune disorders might run together in the same person (Cooper and Stroehla, 2003; Lerner et al., 2015; Fallahi et al., 2019). The most frequent autoimmune disorders are the autoimmune thyroid disorders (AITD), directed against the thyroid gland, whose main clinical features are Graves' disease (GD), and Hashimoto's thyroiditis (HT) (Antonelli et al., 2015). Here we review the new pharmacological progresses made for the treatment of GD, and of Graves' ophtalmopathy (GO).

Grave's Disease and Ophtalmopathy

Graves' disease has a prevalence of about 1–1.5%, in iodine sufficient West countries, with an incidence of 20–30 new cases/100,000 for year. The risk is higher for women, aged 35–55 years, and among African Americans (Smith and Hegedüs, 2016; Kahaly, 2020).

Several factors can predispose to the onset of GD, ranging from genetic, environmental, hormonal conditions to habits such as the smoke. Studies involving twins reinforced the role covered by genetic. A cohort study of 110,814 twins examined co-aggregation and heritability of HT and GD. They observed a higher co-aggregation in monozygotic twins with respect to dizygotic twins, and found a high heritability for GD (Skov et al., 2020). Some variants of the *DRB1*, *DQA1* and *DQB1* genes of the human leukocyte antigen (HLA) class II genes are predictors of the development of GD, whereas others have a protective role, *HLA-DRB1* 07*, *HLA-C03*, and *HLA-C*16* (Vejrazkova et al., 2018; Wémeau et al., 2018). Other immune-competent genes whose variants may be involved in GD are *PTPN22*, *CTLA4*, *CD40*, *FOXP3*, *ARID5*, *NRXN3*, *IKZF3*. Also, other specific thyroid antigens such as "thyroid-stimulating-hormone receptor" (TSH-R), or *Thyroglobulin* (Tg), have been identified by a whole-genome linkage study as major AITD risk genes (Tomer et al., 2003; Vejrazkova et al., 2018). Susceptible individuals could be more easily influenced by environmental triggers, such as external radiation, iodine, selenium, smoking or viruses (Ferrari et al., 2017). Lately, 5 cases of GD reappearance, and a case of GO, after SARS COV-2 infection has been observed (Mateu-Salat et al., 2020; Harris and Al Mushref, 2021; Jiménez-Blanco et al., 2021; Lanzolla et al., 2021).

Therefore, these conditions predispose to the break of the immune tolerance towards thyroid antigens, mainly against the TSH-R. Anti-TSH-R autoantibodies (TRAb) are implicated in the thyroidal and extra-thyroidal manifestations of GD. TRAb are released by B lymphocytes, that infiltrate the thyroid gland during the autoimmune process. They are functionally divided in stimulating (TSAb), blocking (TBAb) and neutral antibodies, with the stimulating ones that induce the hyper-production of thyroid hormones, therefore leading to the clinical manifestations of hyperthyroidism (Smith and Hegedüs, 2016; Antonelli et al., 2020). TSAb have a significant role not only in the thyroid gland, but also in the extra-thyroidal manifestations of GD, such as GO, or pretibial myxedema. Other thyroid antigens are involved in the autoimmune process of GD, such as thyroid peroxydase (TPO) and/or Tg, whose antibodies are found in about 50–70% of cases of GD (Wémeau et al., 2018). The involvement of autoantibodies binding the insulin-like growth factor-1 receptor (IGF-1R) has been found to be implicated in the development of GO (Smith, 2019). They are able to induce the expression of the chemokine "regulated on activation normal T cell expressed and secreted" (RANTES) and IL-16 (Pritchard et al., 2002; Pritchard et al., 2003; Smith, 2019) attracting T lymphocytes, that enter into the site of tissue damage inducing and perpetuating the inflammatory process (Cruikshank et al., 1987; Schall et al., 1988; Smith, 2019).

Cytokines/Chemokines in GD

Fundamental is also the role covered by "Th1 chemokines" (CXCL10, CXCL9, CXCL11), and their (C-X-C)R3 receptor in the immune-pathogenesis of both disorders. In the active phase of GD prevails a Th1 immune response, in which, subsequently to a CXCL10 production by resident follicular epithelial cells, occurs a recruitment of Th1 cells. This process leads to the initiation, and amplification of the inflammation (Romagnani et al., 2002; Antonelli et al., 2020) **(Table 1).**

In basal condition thyroid follicular cells do not secrete Th1 chemokines, while a release occurs under interferon (IFN)-γ, and it is higher under a combined IFN-γ and tumor necrosis factor (TNF)-α (IFN-γ+TNF-α) stimulation (Antonelli et al., 2006a; Antonelli et al., 2009; Antonelli et al., 2010; Ferrari et al., 2015; Fallahi et al., 2020). Therefore, the cytokines stimulation made thyrocytes largely involved in the inflammatory process through the release of Th1 chemokine. The peroxisome proliferator-activated receptor (PPAR)-γ agonists, such as PPAR-α agonists, instead inhibit this process (Antonelli et al., 2009; Antonelli et al., 2010; Antonelli et al., 2011; Ferrari et al., 2015).

Both the active and the relapse phase of GD is characterized by high circulating Th1 chemokines, that decline with methimazole (MMI) therapy. The immune-modulatory effect of MMI is associated with the decrease of serum CXCL10, that achieves normal levels with thyroid hormones normalization, or with GD in remission. The reduction of circulating CXCL10 was not associated with the reduction of AbTg or AbTPO levels, but with the decrease of TRAb (Antonelli et al., 2006a; Antonelli et al., 2006b; Antonelli et al., 2013).

CXCL10 serum levels were also assessed in GD patients who underwent total thyroidectomy or radioactive iodine (RAI) treatment. The decrease of CXCL10 levels following these treatments suggests that the site of production of this chemokine is the thyroid gland itself (Antonelli et al., 2006c; Antonelli et al., 2007; Leite et al., 2011).

Furthermore, a study investigated CXCL10 levels in subjects with: 1) 16 new diagnoses of GD in therapy with MMI; 2) 15 relapsed GD in treatment with RAI; 3) 18 controls. Subjects treated with MMI reported a decline of CXCL10 and euthyroidism after 6 and 12 months; those treated with RAI showed a reduction of CXCL10 levels after 3, 6, 9, and 12 months, with a similar TRAb decrease (Leite et al., 2011).

Cytokines/Chemokines in GO

The onset of GO and GD are often concomitant, with GO involving almost 30–50% of GD patients. Subjects who are more prone to develop a GO are smoker, or patients with a severe hyperthyroidism, and those with very high levels of TSAb. Athough a primary prevention of GO is not available, the progression from a subclinical condition into overt and/or

TABLE 1 | Cytokines and/or Chemokines in Graves' disease and in Graves' Ophtalmopathy.

Cytokines and/or chemokines	Studies [ref]
IL-6	-sIL-6R concentrations were higher in GD patients with active inflammatory thyroid-associated ophthalmopathy than those in patients with inactive or absent thyroid-associated ophthalmopathy Salvi et al. (1996)
TNF-α/IFN-γ	-IFN-γ, or IFN-γ+TNF-α combination stimulate Th1 chemokines in TFCs Antonelli et al. (2006a); Antonelli et al. (2009); Antonelli et al. (2010); Ferrari et al. (2015); Fallahi et al. (2020) -IFN-γ, or IFN-γ+TNF-α combination stimulate Th1 chemokines in the primary cell cultures of retro-bulbar cells of GO patients Antonelli et al. (2006a); Dong et al. (2011)
CXCL10/CXCL9	-Maximal expression of CXCL10 and CXCL9 was found in the thyroid gland of patients with recent-onset GD and was correlated with IFN-γ. High levels of CXCL10 could be measured in the serum of patients with short-duration GD Romagnani et al. (2002) -Significant reductions in CXCL9 and CXCL10 serum concentrations during CS and TR treatment as compared both to control group and to basal values in GO patients Mysliwiec et al. (2012)
CXCL10	-Thyrocytes and retrobulbar cell types participate in the self-perpetuation of inflammation by releasing chemokines under the influence of cytokines. PPAR-γ activation plays an inhibitory role in this process Antonelli et al. (2006a) -sCXCL10 are associated with the active phase of GD in both newly diagnosed and relapsing hyperthyroid patients. The reduction of sCXCL10 in treated patients with GD may be related to the immunomodulatory effects of MMI Antonelli et al, (2006b) - sCXCL10 are higher in newly diagnosed hyperthyroid patients with GD than in those with TNG, and decrease when euthyroidism is achieved with antithyroid therapy Antonelli et al. (2006c) - High sCXCL10 are associated with the hyperthyroid phase in GD but not TNG Antonelli et al. (2007) - Data show a relationship between serum CXCL10 and GD activity Leite et al. (2011) -CXCL10 participates in the early inflammatory response after radioactive iodine therapy in patients with GD and shows a strong association with the autoimmune process Dong et al., 2011
CXCL9/CXCL11	-Thyrocytes and retrobulbar cell types from patients with GD and GO released CXCL9 and CXCL11 chemokines when stimulated with cytokines. PPAR-γ activation plays an inhibitory role in this process Antonelli et al. (2009) - PPAR-α has been found in GD and control thyrocytes. PPAR-α activators are potent inhibitors of the secretion of CXCL9 and CXCL11 Antonelli et al. (2010) -Serum CXCL9 and CXCL11 levels are associated with the active phase of GD both in newly diagnosed and relapsing hyperthyroid patients. The reduction of serum CXCL9 and CXCL11 levels in GD patients in treatment with MMI, may be related to the immunomodulatory effects of MMI Antonelli et al. (2013) -PPAR-α activators inhibit CXCL9 and CXCL11 chemokines in normal and GO fibroblasts and preadipocytes Antonelli et al. (2012).
CXCL10/CCL2	-CCL2 is modulated by IFN-γ and TNF-α in GD and normal thyrocytes. PPAR-α activators inhibit the secretion of CXCL10 and CCL2 in thyrocytes Antonelli et al. (2011) -EOM participates in the self-perpetuation of inflammation by releasing CXCL10 and CCL2 chemokines under the influence of cytokines, in GO. PPAR-γ agonist activation plays an inhibitory role on CXCL10, but stimulates the release of CCL2 Antonelli et al. (2014)

CS, Corticosteroids; EOM, extra-ocular muscle; GD, Graves' disease; GO, Graves' Ophtalmopathy; IFN, Interferon; IL, interleukin; MMI, methimazole; PPAR, peroxisome proliferator-activated receptor; sCXCL10, Serum levels of CXCL10; TFCs, thyroid follicular cells; TNG, toxic nodular goitre; TR, teleradiotherapy.

severe ones can be avoided through an early diagnosis, an accurate control of thyroid function, stop of smoking, and with the early therapy of mild GO (Wiersinga and Bartalena, 2002; Perricone et al., 2016; Smith et al., 2017) **(Table 1)**.

GO retro-bulbar cells (fibroblasts and preadipocytes) are highly involved in the perpetuation of the orbital inflammation by releasing Th1 chemokines (CXCL10, CXL11, CXCL9) under the influence of IFN-γ. Higher serum CXCL10 levels have been observed in GO patients with active disease in comparison to the inactive ones. Moreover, Th1 chemokines were basally absent in the primary cell cultures of retro-bulbar cells of GO patients; whereas their release was stimulated by IFN-γ, or IFN-γ+TNF-α stimulation (Antonelli et al., 2006a; Dong et al., 2011). PPAR-α, -δ, and -γ are found in GO fibroblasts or preadipocytes, and the PPAR-γ agonists showed an inhibitory role on Th1 chemokines release (Antonelli et al., 2009; Antonelli et al., 2012).

Another study explored the involvement of retro-bulbar myoblasts in the immune-pathogenesis of GO (Antonelli et al., 2014). High serum CXCL10 levels have been observed in both patients having active GO associated with extraocular muscle (EOM) or with orbital fat involvement, in comparison with controls. CXCL10 was not detectable in primary EOM cells from GO patients, whereas it was released under the cytokines stimulation (IFN-γ and/or TNF-α). Therefore, EOM are involved in the inflammatory GO process through the release of Th1 chemokines (Antonelli et al., 2014).

A potential use of Th1 chemokines as markers of GO activity has been investigated by a study that involved forty-two GO subjects of which: 20 were GD patients (half in euthyroidism and half in hyperthyroidism); 15 GO patients in euthyroidism [previously treated with intravenous of methylprednisolone (ivMP) and teleradiotherapy], and seven were controls. Interestingly, a significant decrease of Th1 chemokines

occurred after ivMP and teleradiotherapy. The reduction of circulating Th1 chemokines was not associated with the reduction of AbTg or AbTPO levels, nor of TRAb.

Therefore, these chemokines may aid in the therapeutic decision-making of GO patients (Mysliwiec et al., 2012).

THERAPY FOR GD

Antithyroid Drugs

MMI, carbimazole, and propylthiouracil (PTU) are the first-choice therapy for GD. These drugs act by inhibiting TPO, and blocking the synthesis of thyroid hormones. PTU also blocks extrathyroidal deiodination of T4 to T3. The toxicity profile of these drugs makes them the preferred with respect to radioiodine (Bartalena et al., 2016; Burch and Cooper, 2015; Antonelli et al., 2020), however the risk of relapse after therapies is high. Furthermore, MMI and PTU have immune-modulatory effect reducing TSAb levels (Antonelli et al., 2013).

Radioiodine Therapy

RAI has been widely used; it gives relief from symptoms of hyperthyroidism within weeks. Antithyroid drugs can be suspended 3–7 days before and after radioiodine in order to improve its effectiveness. However, radioiodine can cause or worsen GO. Therefore, a close monitoring of the thyroid function should be performed, and when hypothyroidism occurs, it needs to be treated as soon as possible (Galetta et al., 2008; Smith and Hegedüs, 2016).

Surgery

Surgery is needed in particular conditions, such as if the patient do not want to receive anti-thyroid drugs, or radioiodine; in presence of a large goiter; and for women who would like to have pregnancy. The patients must reach euthyroidism, before they can undergo surgery. This will reduce the risk of complications (Feroci et al., 2014; Smith and Hegedüs, 2016).

Antigen-specific Immunotherapy

The antigen-specific immunotherapies aim to re-establish an immunological tolerance against the immune dominant epitopes involved in autoimmunity, without inducing generalized immunosuppression (Pearce et al., 2019). A study investigated a combination of two TSHR peptides (ATX-GD-59) in 12 subjects with mild-to-moderate untreated hyperthyroidism. A potential efficacy of this treatment has been suggested; 70% of the treated subjects reported an improvement in free thyroid hormones (Pearce et al., 2019).

THERAPY FOR GO

Corticosteroids Therapy

The common treatment for active GO are high-dose of ivMP. A multicenter trial demonstrated the effectiveness of ivMP in improving inflammation in about 80–70% of the cases, and eye muscle function in 50%. Nevertheless, about 20% were no significantly responders to the treatment, and progression disease or compression of the optic nerve occurred in about 4% of the subjects (Bartalena et al., 2012).

Therefore, new targets involved in the autoimmune reaction have been taken in accounts for the development of new drugs, such as TSH-R, the IGF-1R (on fibroblasts), T or B lymphocytes, chemokines and cytokines (Fallahi et al., 2016).

TSH-R Antagonists

Drugs acting against TSH-R have been recently investigated. Promising results have been obtained by a molecule NCGC0022960 able to reduce the production of hyaluronic acid in primary cell culture of retro-orbital fibroblasts/adipocytes of GO (Emerson, 2011; Turcu et al., 2013).

In a patients with follicular thyroid cancer (FTC), GD, with high levels of TSAb, and severe GO, was tested K1-70 a monoclonal antibody anti-TSHR. After the start of the therapy, the TSAb activity decreased and GO improved. Moreover, on K1-70 monotherapy during the pause in lenvatinib, used for the treatment of FTC, occurs a stabilization of several metastatic lesions (Ryder et al., 2021).

Etanercept and Tocilizumab

Cytokines are largely involved in the autoimmune process of the GO. TNF-α and IL-6 have a crucial role in this process (Bahn, 2010).

Etanercept is a dimeric protein able to bind two molecules of TNF, avoiding its interaction with receptors on the cell surface, and subsequently the TNF-mediated inflammatory responses. This molecule is the choice option for different autoimmune disorders [e.g. rheumatoid arthritis (RA), ankylosing spondylitis in adults, and juvenile idiopathic arthritis or plaque psoriasis in paediatric patients] (Scott, 2014). In a pilot study the efficacy of etanercept was investigated in 10 GO subjects (25 mg twice weekly, were administered for 12 weeks). An improvement was observed in 60% of patients; a reactivation of GO occurred in three patients after cessation. No serious adverse events (AEs) or side effects were registered during a follow-up of 18 months (Paridaens et al., 2005). Another paper reported a case of a patient with RA and GO. She was treated with etanercept for RA achieving also a clinical improvement of GO symptoms (Boskovic et al., 2019). Additional researches are needed in order to evaluate the effectiveness of TNF-α inhibitors, and to compare its side effects with the current medical treatment.

The cytokine IL-6 is released by T lymphocytes and macrophages and has a pro-inflammatory activity. GO patients in the active phase showed increased levels of circulating IL-6 and of its receptor (Salvi et al., 1996). The monoclonal antibody (mAb) tocilizumab (TCZ) acts against the IL-6 receptor, and received the approval for the treatment of RA, systemic juvenile idiopathic arthritis and Castleman's disease (Emery et al., 2008; Yokota et al., 2008). TCZ was evaluated in 18 GO patients, not responders to corticosteroids (CS). Thirteen patients showed a decreased proptosis, fifteen had an improvement of the extraocular motility and seven out of 13 resolved their diplopia (Pérez-Moreiras et al., 2014). Another open-label multicenter study assessed the effectiveness of TCZ

enrolling 48 patients with glucocorticoid-resistant GO (Sánchez-Bilbao et al., 2020). The follow-up lasts for a mean of 16.1 ± 2.1 months, and it was observed a decrease of disease activity [Clinical Activity Score (CAS) ≤ 3] in many patients; TCZ was withdrawn in 29 cases, because of low disease activity in 25 cases, or inefficacy in four subjects. No serious AEs were registered. Thereby, TCZ appears an efficacy, useful and safe therapeutic option in refractory GO treatment (Sánchez-Bilbao et al., 2020).

Rituximab

Rituximab (RTX) acts against CD20 placed on B cells; thereby it induces B cells death, and is indicated in the therapy of those diseases characterized by elevated levels of B-lymphocytes or dysfunctional B-lymphocytes, and overactive B-cells. This mAb has no effect on plasma cells, it doesn't interfere with the antibody synthesis (Ahuja et al., 2008). Since RTX reduces the number of B lymphocytes, the burden of cytokines and the secreted autoantibodies, it has been suggested for the treatment of GO (Salvi et al., 2012). Conflicting results were reported about the efficacy of RTX in GO.

A study included 25 GO subjects in a prospective, placebo-controlled, randomized trial (Stan et al., 2015); patients received two RTX infusions, or two saline infusions, 2 weeks apart. RTX appeared not effective in GO, because no differences were registered about the improvement of CAS with respect to placebo (Stan et al., 2015).

However, another double-blind, randomized trial enrolling 32 subjects reported different outcomes. The patients received RTX or ivMP; 100% of RTX patients achieved an improvement at 24 weeks, compared to 69% after ivMP, therefore assessing a higher efficacy of RTX than ivMP in GO patients (Salvi et al., 2015).

Recently a multicenter retrospective study (Deltour et al., 2020) investigated the efficacy of RTX in forty GO patients resistant to CS, or in cases of CS dependence. The Authors found that RTX is effective as a second-line treatment of these patients, especially if the disease is recent and active; and when it is administered in the early phase of the disease. The time of administration may explain the contradictory results obtained in the previous randomized studies (Salvi et al., 2015; Stan et al., 2015; Deltour et al., 2020).

Teprotumumab

An overexpression of IGF-1R has been found in orbital connective tissues, T and B cells in GD and GO. GD patients generated autoantibodies that are able to bind to IGF-1R and initiate the signaling from the TSHR/IGF-1R physical and functional protein complex. Therefore, the use of mAbs against IGF-1R may attenuate signaling from either TSHR or IGF-1R (Smith, 2021).

Teprotumumab (RV 001, R1507) is a human monoclonal anti-IGF-1R blocking antibody. An *in vitro* study, showed its efficacy in reducing the fibrocyte display of IGF-1R and TSH-R, such as their downstream signals, blocking the induction of pro-inflammatory cytokines (Chen et al., 2014).

A first multicenter, double-masked, randomized, placebo-controlled trial was carried out to investigate the efficacy of teprotumumab in patients with active, moderate-to-severe ophthalmopathy (Smith et al., 2017). The 88 enrolled subjects were randomly assigned to the placebo group or to the teprotumumab group. 69% of patients of the teprotumumab group had a response at week 24 ($p < 0.001$), with respect to the 20% of the placebo group. Moreover, the response was rapidly achieved ($p < 0.001$) in the teprotumumab group, 43% at week 6, against only 4% of the placebo. These findings supported the efficacy of this drug in reducing proptosis and CAS in patients with active GO (Smith et al., 2017).

A subsequent randomized, double-masked, placebo-controlled, phase 3, multicenter trial, involved 83 patients with moderate to severe GO (with a duration of GO < 9 months), of whom 41 received teprotumumab and 42 placebo. Teprotumumab led to better outcomes in proptosis, CAS, diplopia, and quality of life than placebo; serious AEs were uncommon (Douglas et al., 2020). Additionaly, the non responders were included in an extension of the phase 3 trial; they received teprotumumab as an open label, regardless of whether or not they had received the active drug, or placebo, during the 24-weeks treatment phase (Smith, 2021). The response to the therapy occurs in a similar fraction of patients, such as in the initial intervention. Follow-up data (of phase 3 trial, plus extension study) revealed that the majority of patients who responded with amelioration of proptosis and diplopia at week 24 maintained their responses (56 and 58%, respectively) (Smith, 2021).

These studies showed the efficacy of teprotumumab as well as its safety. The AEs encountered were mild to moderate in severity, with the most common being hyperglycemia found especially in patients with diabetes, and easily managed by adjusting the therapy. Other AEs were muscle cramps, hearing abnormalities, hair loss, diarrhea and dysgeusia (Chen et al., 2014; Smith et al., 2017; Smith, 2021).

Teprotumumab has been approved by the US FDA for the therapy of GO, and is now in clinical use in North America (Smith, 2021).

However, more studies are needed to assess its effectiveness in such conditions e.g. patients with a chronic and less active GO condition or with an impaired vision due to compressive optic neuropathy (Markham, 2020; Smith, 2021).

CONCLUSION

Graves' disease (GD) is a condition caused by an autoimmune process involving the thyroid gland, whose main outcome is hyperthyroidism. TSAb start the autoimmune process stimulating the overproduction of thyroid hormones. In addition, TSAb can stimulate TSH-R expressed in fibroblasts and orbital pre-adipocytes, leading to the manifestation of GO. Also, autoantibodies directed against IGF-1R have an important role in immune-pathogenesis of GO. Fundamental is the role played by cytokines (IFN-γ, TNF-α, Il-6), and Th1 chemokines in the immune-pathogenesis of both disorders, particularly in the active phase.

Novel discoveries **(Table 2)** in the field led to the investigation of promising therapies, such as immune-therapies towards

TABLE 2 | Latest drugs for Graves' Ophtalmopathy.

Drugs	Molecular targets	Studies [ref]
NCGC0022960	TSH-R	Reduced production of hyaluronic acid in primary cell culture of retro-orbital fibroblasts/adipocytes of GO Turcu et al. (2013)
K1-70	TSH-R	-Case report; TSAb activity decreased and GO improved Ryder et al. (2021)
Etanercept	TNF-α	-Pilot study; improvement in 60% of pts Paridaens et al. (2005) -Case report patient with RA and GO: clinical improvement of GO symptoms Boskovic et al (2019)
Tocilizumab	IL-6	- 18 pts, not responders to corticosteroids. Thirteen pts showed a decreased proptosis, fifteen had an improvement of the extraocular motility and 7 out of 13 resolved their diplopia Pérez Moreiras et al., (2014) -An open-label multicenter study including 48 pts with glucocorticoid-resistant GO. Decrease of disease activity was registered in many pts; TCZ was withdrawn in 29 pts, because of low disease activity in 25 cases, or inefficacy in 4 subjects. Sánchez-Bilbao et al. (2020)
Rituximab	CD20 on B cells	-25 GO subjects in a prospective, placebo-controlled, randomized trial; RTX appeared not effective in GO, because no differences were registered about the improvement of CAS with respect to placebo Stan et al. (2015) - A double-blind, randomized trial enrolling 32 subjects. The pts received RTX or ivMP; 100% of RTX pts achieved an improvement at 24 weeks, compared to 69% after ivMP Salvi et al. (2015) -A multicenter retrospective study involving forty GO pts resistant to CS, or having CS dependence. RTX appeared effective as a second-line treatment of these pts, especially if the disease is recent and active; and when it is administered in the early phase of the disease Deltour et al. (2020)
Teprotumumab	IGF-1R	-A first multicenter, double-masked, randomized, placebo-controlled trial involved pts with active, moderate-to-severe ophthalmopathy. The 88 enrolled subjects were randomly assigned to the placebo group or to the teprotumumab group. 69% of pts of the teprotumumab group had a response at week 24 ($p < 0.001$), with respect to the 20% of the placebo group. The response was rapidly achieved ($p < 0.001$) in the teprotumumab group, 43% at week 6, against only 4% of the placebo. These findings supported the efficacy of this drug in reducing proptosis and CAS in pts with active GO Smith et al. (2017) -A randomized, double-masked, placebo-controlled, phase 3, multicenter trial, involved 83 pts with moderate to severe GO; 41 received teprotumumab and 42 placebo. Teprotumumab led to better outcomes in proptosis, CAS, diplopia, and quality of life than placebo; serious AEs were uncommon Douglas et al. (2020) The non responders were included in an extension of the phase 3 trial; they received teprotumumab as an open label, regardless of whether or not they had received the active drug, or placebo, during the 24-weeks treatment phase Smith. (2021). The response to the therapy occurs in a similar fraction of pts, such as in the initial intervention. Follow-up data (of phase 3 trial, plus extension study) revealed that the majority of pts who responded with amelioration of proptosis and diplopia at week 24 maintained their responses (56 and 58%, respectively) Smith. (2021)

AEs, Adverse Events; CAS, Clinical Activity Score; CS, Corticosteroids; GO, Graves' Ophtalmopathy; ivMP, Intravenous Methylprednisolone; Pts, Patients; RA, Rheumatoid Arthritis; RTX, rituximab; TCZ, tocilizumab; TNF, Tumor Necrosis Factor TSAb, Thyroid Stimulating Antibodies; TSH-R, Thyroid-stimulating-hormone Receptor.

specific antigens (for example against TSH-R), aiming in restoring the immune tolerance in GD.

After the initial attempt of biologic therapies in GO (Antonelli et al., 1992; Baschieri et al., 1997), more recently, etanercept (that blocks the TNF-mediated inflammatory responses), TCZ (that acts against the IL-6 receptor), and RTX (that acts against CD20) have proven to be useful and safe therapeutic options in refractory GO treatment. Furthermore, teprotumumab (a human monoclonal anti-IGF-1R blocking antibody), has been revealed effective in the treatment of patients with moderate-severe GO and it is now approved for GO therapy in United States.

More, extensive researches are needed to deepen out these drugs as well as to identify new targeted and effective therapies, that will permit a more precise identification of GD, or GO, patients able to respond to specific targeted therapies.

AUTHOR CONTRIBUTIONS

GE, PF, AA and SMF conceived the paper. All authors reviewed and approved the final version of the manuscript.

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Precision Medicine in Autoimmune Thyroiditis and Hypothyroidism

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Autoimmune thyroid diseases (AITD) are T-cell-mediated organ specific autoimmune disorders, deriving from an altered response of the immune system that leads to the immune attack to the thyroid. Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the two principal AITD clinical presentations. Hypothyroidism and thyrotoxicosis are, respectively, the clinical hallmarks of HT and GD. Patients with autoimmune thyroiditis are treated daily with synthetic L-thyroxine (L-T4) at the dose of 1.5–1.7 μg/kg. Various L-T4 formulations are commercially available (tablet, liquid solution, or soft gel capsule). L-T4 in tablets is generally prescribed to treat hypothyroidism, whereas the liquid formulation, or soft gel capsules, can be administered in hypothyroid patients in case of malabsorption or in patients in therapy with drugs interfering with L-T4 absorption. Furthermore, myoinositol has a crucial role in thyroid autoimmunity and function. Clinical studies reported a significant decline in TSH and antithyroid autoantibodies levels after treatment with myoinositol + selenium in patients with subclinical hypothyroidism and autoimmune thyroiditis. Moreover, thyroidectomy can be rarely recommended in patients with autoimmune thyroiditis, with cosmetic reasons for a goiter, or with important signs or symptoms of local compression, or nodular disease with a "suspicious" cytology for malignancy. Furthermore, a recent randomized trial suggested that total thyroidectomy can improve quality of life and fatigue, while medical therapy did not. In this review, we overview currently available evidence in personalized medicine in patients with autoimmune thyroiditis and hypothyroidism. Further research is needed in larger population to investigate the effect of these new treatments on quality of life.

Keywords: Hashimoto's thyroiditis, autoimmune thyroid disorders, autoimmune thyroiditis, hypothyroidism, levothyroxine, thyroidectomy

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INTRODUCTION

Autoimmune thyroiditis (AT) and Graves' disease (GD) are the main autoimmune thyroid disorders (AITD), which are the most common autoimmune disorders (Romagnani, 1998; Antonelli et al., 2015).

AITD are distinguished by the breakdown of tolerance of the immune system against the thyroid (Antonelli et al., 2015). GD and AT are clinically characterized by thyrotoxicosis and hypothyroidism,

respectively, by infiltrative autoreactive lymphocytes in the gland and the presence of serum antithyroid autoantibodies (ATA; Antonelli et al., 2015).

In the general population, women have a higher risk to develop AITD than men (~4–8/1); the prevalence of AITD changes geographically, and it is more elevated in areas with iodine sufficiency (Antonelli et al., 2015). In the presence of AT, the frequency of hypothyroidism increases with age, such as the frequency of ATA (Antonelli et al., 2015).

Hashimoto's thyroiditis (HT) leads to a chronic inflammation of the thyroidal tissue (McLeod and Cooper, 2012; Antonelli et al., 2015), wherein hypothyroidism is present in ~25% of patients (McLeod and Cooper, 2012; Caturegli et al., 2014; Antonelli et al., 2015; Ragusa et al., 2019), and it was first described with significant signs of AT, lymphocytic infiltration, atrophy of follicular cells, fibrosis, and goiter. Thyroid hormones (TH) affect different organs and tissues, and for this reason, the symptoms and signs of hypothyroidism are various and unspecific, and can influence different systems (i.e., pulmonary, cardiovascular, hematopoietic, urinary, gastrointestinal, and reproductive; Galetta et al., 2008; Iddah and Macharia, 2013; Caturegli et al., 2014; Ragusa et al., 2019).

AITD are generally associated with another autoimmune disease [i.e., Sjogren syndrome (SS), systemic lupus erythematosus (SLE), sarcoidosis, systemic sclerosis (SSc), vitiligo, rheumatoid arthritis (RA), type 1 diabetes mellitus (T1D), celiac disease (CD), autoimmune gastritis, and HCV-related cryoglobulinemia], as reported in ~19% of 3,069 patients with AT vs 2 gender- and age-matched control groups of 1,023 healthy subjects and 1,023 non-toxic multinodular goiter (MNG) subjects (Fallahi et al., 2016a). Moreover, the association of three different autoimmune diseases was reported in AT with respect to controls (Fallahi et al., 2016a). Another study reported similar data in 3,209 GD subjects (984 with Graves' ophthalmopathy) vs 1,069 controls, 1,069 MNG, and 1,069 AT patients, showing the prevalence of another autoimmune disease in ~17% of GD subjects (Ferrari et al., 2019a).

Furthermore, epidemiological studies have reported that AITD can be associated with papillary thyroid cancer (PTC). Elevated TSH levels were associated with the PTC risk in 13,738 AT patients (Fiore et al., 2011). Conversely, other papers demonstrated that both thyroid autoimmunity and high TSH levels are independent risk factors for PTC (Boi et al., 2013).

The association between inflammation and TC involves different components of the immune system (macrophages, lymphocytes, cytokines, and chemokines) (Antonelli et al., 1999; Ferrari et al., 2019b; Ferrari et al., 2019c).

GENETIC SUSCEPTIBILITY AND ENVIRONMENTAL FACTORS

Genetic Susceptibility

Different observations are at the basis of the genetic susceptibility to AITD: (1) the familial clustering (25% of AITD in siblings of AITD subjects); (2) AITD sibling risk ratio of ~17; and (3) a strong prevalence of ATA in siblings of AITD patients (Brix and Hegedüs, 2012).

The association among AITD, the presence of ATA and certain genes [i.e., human leukocyte antigen (HLA), IL2RA, CTLA4, and PTPN22], has been demonstrated, and also other AITD genes were identified by genome-wide association studies (GWAS) [i.e., HLA class I, and TSH receptor (TSH-R)] (Simmonds, 2013; Tomer and Davies, 2013). Other AITD risk genes have been identified by GWAS and Immunochip techniques (i.e., FOXE1, BACH2, RNASET2 and GDCG4p14; Simmonds, 2013).

Of particular interest, 7/11 known susceptibility genes are involved in the role of T cells, suggesting their importance in the immune-pathogenesis of AITD (Antonelli et al., 2015), even if chronic AT can occur in the absence of serum ATA (Rotondi et al., 2014a). A case-control retrospective study enrolled 55 patients with serum negative chronic AT and 110 patients with chronic AT. Patients with serum negative chronic AT had significantly lower mean TSH levels, higher mean FT4 levels, comparable FT3 levels, and a significantly lower mean thyroid volume vs patients with chronic AT. The data suggested that patients with serum negative chronic AT would display a milder clinical phenotype (Rotondi et al., 2014b).

Furthermore, a monogenic form of AITD was first reported in a family with autosomal dominant inheritance of HT (Lo et al., 2018).

Environmental Factors

In iodine sufficient areas, hypothyroidism is due mainly to HT. A lower AITD prevalence is shown in iodine-deficient areas, whereas an exaggerated iodine intake is associated with a higher AITD prevalence (Iddah and Macharia, 2013; Ferrari et al., 2017a).

Smoking habits are a risk factor for Graves' hyperthyroidism (Perricone et al., 2016); whereas, overall, Graves' ophthalmopathy reduce the risk of hypothyroidism (Carlé et al., 2012).

Radiation exposure is another risk factor, and elevated ATA levels have been reported in children after nuclear disasters, with a subsequent raised risk of TC and thyroid dysfunctions (Antonelli et al., 1996).

The role of viruses in the pathogenesis of AITD has been evaluated with contrasting findings; however, an association between AITD and hepatitis C virus chronic infection (HCV) has been confirmed (Menconi et al., 2011; Ferrari et al., 2013; Ferri et al., 2015; Zignego et al., 2015; Ferrari et al., 2020).

Also some drugs (i.e., tyrosine kinase inhibitors) are an emerging cause of primary hypothyroidism (Fallahi et al., 2014).

Selenium and vitamin D deficiency is a possible AITD risk factor, too. The thyroid expresses specific selenoproteins, and a decreased selenium assumption is an AITD risk factor (Duntas, 2010; Wang et al., 2018; Benvenga et al., 2020a).

Another AITD risk factor is stress, both emotional and psychological, that perhaps owing to the effect of cortisol on immune cells, followed by immune hyperactivity, can lead to thyroid autoimmunity (Iddah and Macharia, 2013).

LEVOTHYROXINE TREATMENT

The synthetic hormone L-T4 is recommended as therapy of hypothyroidism-related conditions as it has a chemical structure similar to T4 (Miccoli et al., 1993; Fallahi et al., 2017a).

The tablet formulation of L-T4 contains the stable salt sodium L-T4, along with various excipients, and it needs an acid gastric pH to be dissolved (Centanni et al., 2006; Virili et al., 2019a). In the absence of factors that alter L-T4 absorption, ~70% of tablet L-T4 is absorbed into the duodenum and jejunum (Fallahi et al., 2017a).

Thanks to more sensitive TSH assays, nowadays, 1.5–1.7 μg/kg body weight is the ideal daily L-T4 replacement dose, able to obtain normal TSH levels in most hypothyroid subjects (Caturegli et al., 2014). In particular, in patients with serum negative chronic AT, the required dose of L-T4 appears to be lower, as reported by a study conducted in 49 hypothyroid patients with serum negative chronic AT and in 98 hypothyroid patients with HT (Croce et al., 2020).

In spite of this, owing to different interfering issues (Miccoli et al., 1993), ~20–50% of patients do not respond to the L-T4 treatment (Eligar et al., 2016; Virili et al., 2019b) and require a higher dose and monitoring (Ernst et al., 2017). Once excluded, a possible pseudomalabsorption linked to a scarce compliance with the prescribed regimen, gastrointestinal disorders, or interfering drugs can cause a decrease in intestinal absorption of L-T4 and are considered the main cause of refractory hypothyroidism (Virili et al., 2019b).

Levothyroxine Tablets Malabsorption

L-T4 tablets are usually taken before breakfast. It has been shown that the assumption of L-T4 10 min before coffee in the morning reduces its absorption (Benvenga et al., 2008). A prospective, open-label, randomized, cross-over study compared the L-T4 administration during fasting with that during breakfast. In patients receiving L-T4 during breakfast, TSH was more elevated than in those during fasting (2.89 vs 1.9 mIU/L), suggesting that it is better to take L-T4 during a fasting state (Perez et al., 2013).

Moreover, different intestinal or gastric diseases can alter the L-T4 tablet absorption (Formenti et al., 2015). For this reason, in patients affected by *Helicobacter pylori* (HP) gastritis, or atrophic gastritis, who have an altered acid secretion, the daily L-T4 requirement is raised by 22–34% (Annibale et al., 1997; Yao and Forte, 2003; Centanni et al., 2006).

Proton-pump inhibitors (PPI) can reduce L-T4 absorption, too. The effect of PPI on serum TSH in 37 euthyroid subjects who had been administered with stable L-T4 for at least 6 months was evaluated. From before the PPI treatment to 2 months after it, the mean change in TSH was higher than in controls. The data indicated that in hypothyroid patients treated with L-T4 and PPI, further TH measurement and/or adjustment of the L-T4 dose might be necessary (Sachmechi et al., 2007).

Intestinal disorders can lead to an increased need for L-T4, too (Rostom et al., 2006). The association of different autoimmune disorders is well-known (Galetta et al., 2008; Formenti et al., 2015; Fallahi et al., 2016a), and CD and AITD are cognate disorders (Fallahi et al., 2019).

The L-T4 dose in 35 hypothyroid patients with atypical CD and AITD has been evaluated vs patients with AITD only. The dosage should be increased up to 50% if CD patients did not follow a strict gluten-free diet (Virili et al., 2012).

Lactose intolerance (LI) can lead to a reduction of L-T4 absorption (Asik et al., 2014). The L-T4 dose to normalize TSH was evaluated in 34 AITD hypothyroid patients with LI who did not follow a lactose-free diet. Moreover, in another study in LI AITD patients, the dose of L-T4 to normalize TSH was 1.81 μg/kg/day while it was of 1.31 μg/kg/day in AITD patients without LI (Cellini et al., 2014).

Also bariatric surgery can cause reduction of L-T4 absorption (Pirola et al., 2013) as restrictive surgical procedures that increase gastric pH can alter drug dissolution and solubility (Padwal et al., 2010).

Novel Oral Levothyroxine Formulations

Refractory hypothyroidism and the need to increase the "normal dose" of L-T4 (Centanni et al., 2017; Fallahi et al., 2021) has led to the development of new L-T4 preparations, soft gel capsule and the liquid formulation.

Oral Liquid Levothyroxine

The liquid preparation is bioequivalent with the tablet L-T4 (Yue et al., 2012). It contains L-T4, ethanol, and glycerin, and it does not need an acid gastric pH to be dissolved (Vita et al., 2014a).

Two meta-analyses evaluated the efficacy of the liquid formulation: the first one suggested that patients treated with L-T4 tablets with suboptimal TSH levels can obtain the expected TSH upon the switch to liquid L-T4 with the same dose (Virili et al., 2018) and the second one reported that the liquid L-T4 was more effective (in comparison to tablets) in subjects in presence/absence of reduced absorption both in replacement such as in suppressive treatment (Laurent et al., 2018).

Liquid L-T4 has been considered also in 78 newborns with congenital hypothyroidism, reporting a TSH inhibition rate that could be associated with a stronger absorption (compared to tablets; Peroni et al., 2014). Another study confirmed these data (Pirola et al., 2014).

Furthermore, the oral liquid L-T4 can maintain better circulation of normal TSH with respect to tablets also in elderly (Cappelli et al., 2014) and during pregnancy (Cappelli et al., 2015).

Soft Gel Capsule Preparation

In soft gel capsule, sodium L-T4 is in glycerin and water, and maintained into a gelatin matrix. It is free of gluten, lactose, alcohol, dyes, or sugar (Vita et al., 2014a). It is rapidly dissolved in the acid gastric pH.

Another study investigated is if soft gel capsule preparation can bypass the reduced absorption associated with the ingestion of coffee in eight patients, who were switched from the tablets to the capsule for 6 months with the same L-T4 dosage (Vita et al., 2013). Patients followed a proper habit on days 1–90, taking coffee 1 h after the drug assumption, while they followed an

improper habit on days 91–180 taking coffee ≤5 min after the capsule. The obtained data suggested that soft gel capsule is effective in subjects with an improper habit of taking L-T4 (Vita et al., 2013).

Another study evaluated the daily required L-T4 dose in 103 thyroidectomized patients. Even if the L-T4 requirement to attain optimal TSH levels was similar among patients receiving soft gel capsules and tablets, mean TSH decreased by 28% in those receiving the soft gel vs tablets (Di Donna et al., 2018).

Moreover, the effect of the switch from tablets to soft gel capsule preparation was investigated in hypothyroid patients, without increasing the L-T4 dose. In 11/18 patients treated with L-T4 tablets, serum TSH was normal and upon the switch in 16/18 (with a lower median TSH value; Trimboli et al., 2018).

Advances in the Treatment of Hypothyroidism with the New Levothyroxine Preparations

The recently marketed novel preparations of L-T4 have led to a significant reduction in TSH variability in hypothyroid patients, in comparison to tablets.

Novel L-T4 preparations can be administered in hypothyroid patients in case of malabsorption:

- with food and beverages interference, or in subjects who do not wish to ingest L-T4 30–60 min before breakfast (Cappelli et al., 2016; Guglielmi et al., 2018);
- deriving from an increased gastric pH (Centanni et al., 2006; Lahner et al., 2009; Vita et al., 2014b; Lahner et al., 2014; Fallahi et al., 2016b; Cellini et al., 2017; Ribichini et al., 2017; Guzman-Prado et al., 2020);
- following bariatric surgery or with intestinal malabsorption (Pirola et al., 2013; Fallahi et al., 2017b);
- induced by interferent drugs (Fallahi et al., 2014);
- in case of typical or atypical CD (Virili et al., 2012; Zubarik et al., 2015; De Carvalho et al., 2018), or LI (Asik et al., 2014; Fallahi et al., 2017c; Fallahi et al., 2019);
- who can not swallow the tablets (Pirola et al., 2014).

Moreover, both in patients with malabsorptive issues or with no malabsorption, the oral liquid L-T4 is able to maintain more efficiently, than L-T4 tablets, normal TSH values in hypothyroid patients in the long-term follow-up (Antonelli et al., 2021).

MYOINOSITOL AND SELENIUM IN PATIENTS WITH AUTOIMMUNE THYROIDITIS AND HYPOTHYROIDISM

Inositol is a compound soluble in water (Benvenga et al., 2020a), whose most abundant form is myoinositol (Benvenga and Antonelli, 2016).

Myoinositol is the precursor of phosphoinositides, and it takes part into various cellular processes (Fallahi et al., 2018).

In humans, the raised values of TSH were reduced in AT patients with subclinical hypothyroidism after therapy with myoinositol and seleno-methionine, such as AbTg and AbTPO levels. Seleno-methionine alone did not induce the same decrease (Nordio and Pajalich, 2013).

Selenium is an essential micronutrient which is necessary for cellular function, and it exercises its function in the form of the amino acid selenocysteine within selenoproteins (Duntas and Benvenga, 2015).

It is well-known that selenium is determinant in thyroid autoimmunity (Antonelli et al., 2015). Selenium deficiency is associated with an elevated prevalence of AT, owing to a reduced activity of selenium-dependent enzymes within thyrocytes and the immune system (Mazokopakis et al., 2007; Duntas and Benvenga, 2015).

Considering the pathogenetical link between AITD and environmental factors that can trigger oxidative stress and the antioxidant property of selenium, the possible supplementation with sodium selenite or seleno-methionine has been evaluated in AITD (Duntas and Benvenga, 2015). A favorable effect of the combination of myoinositol and seleno-methionine has been shown in patients with subclinical hypothyroidism (Nordio and Pajalich, 2013; Benvenga et al., 2020b).

The immune-modulating action of myoinositol combined with seleno-methionine was investigated in 21 euthyroid AT patients, who received myoinositol in combination with selenium (600 mg/83 μg) tablets, for 6 months twice a day (Ferrari et al., 2017b). After the treatment, TSH levels significantly declined, such as ATA, in particular in AT patients with TSH in the high normal range. These data suggested that myoinositol and seleno-methionine in combination can decrease the risk of a worsening of hypothyroidism. Moreover, after the treatment, also serum CXCL10 chemokine levels declined with respect to basal values, confirming the immune-modulatory effect of combined myoinositol and selenium (Ferrari et al., 2017b).

CXCL10 (or IP-10, the IFN-γ-inducible protein 10) is an IFN-γ-inducible chemokine that is implicated in lymphocyte infiltration and thyroid destruction in HT (Antonelli et al., 2011; Fallahi et al., 2020). The immune-modulatory effect of the combination of myoinositol and selenium on CXCL10 secretion suggests it could reduce the Th1 immune response (Cantrell, 2015).

Another paper evaluated the effect of different additions of myoinositol, seleno-methionine, or their combination on *in vitro* peripheral blood mononuclear cells (PBMC) obtained from three controls and eight HT women, treated with hydrogen peroxide (H2O2; Benvenga et al., 2017). H2O2 alone reduced dose-dependently PBMC proliferation in either groups, and cell vitality by 5% in control subjects and 10% in HT patients, but vitality was rescued by the following additions, inhibiting also the genotoxic effect. Chemokines increased after H2O2 alone, while the following additions dose-dependently diminished these levels, especially with myoinositol + seleno-methionine (Benvenga et al., 2017). Moreover, H2O2 increased the apoptosis in primary thyrocytes and decreased the proliferation, reducing also IFN-γ-induced CXCL10 secretion. The combination of myoinositol + seleno-methionine reduced the cytokine-induced secretion of CXCL10 both in presence/absence of H2O2, while seleno-

methionine alone had no significant effect. This suggested a protective effect of myoinositol in thyrocytes (Ferrari et al., 2018).

An observational and retrospective study evaluated TH (after 6 and 12 months of treatment) in HT patients (both euthyroid, such as with subclinical hypothyroidism) and divided them in: untreated, treated with seleno-methionine alone (83 μg/day), and treated with seleno-methionine + myoinositol (83 μg/day + 600 mg/day). TSH levels were reduced (31–38%) in HT patients treated with seleno-methionine and/or seleno-methionine + myoinositol, while TSH increased in untreated patients. In particular, the TSH decrease was shown earlier in patients receiving seleno-methionine + myoinositol than in those treated with seleno-methionine alone (Pace et al., 2020).

Furthermore, the effect of myoinositol has been investigated in 86 patients with subclinical hypothyroidism and HT, who received 600 mg myoinositol and 83 μg seleno-methionine for 6 months. A significant amelioration in TSH values and in the quality of life of the patients was reported (Nordio and Basciani, 2017a). Another study was conducted in 168 patients with HT and TSH between 3 and 6 mIU/mL, who were subdivided into two groups: treated with myoinositol + seleno-methionine (600 mg + 83 μg, respectively) and treated with seleno-methionine (83 μg). TSH, FT4, AbTPO, and AbTg improved only in patients treated with myoinositol + seleno-methionine (Nordio and Basciani, 2017b).

A placebo-controlled randomized prospective study evaluated the short-term effect of L-seleno-methionine on the thyroid function in 76 euthyroid HT patients; among them, 38 received L-seleno-methionine (166 μg/die) and 38 received placebo for 6 months. TSH, FT4, FT3, AbTPO, thyroid echogenicity, and CXCL10 were not statistically different between the two groups of patients at time 0 after 3 and 6 months. The data suggested that the short-term L-seleno-methionine supplementation has a slight impact on the natural course in euthyroid HT (Esposito et al., 2017).

THE ROLE OF THYROIDECTOMY IN AUTOIMMUNE THYROIDITIS AND HYPOTHYROIDISM

Thyroidectomy can be rarely recommended in AT patients, with cosmetic reasons for a goiter, or with important signs or symptoms of local compression, or nodular disease with a "suspicious" cytology for malignancy.

Moreover, in some patients with HT, symptoms persist despite their euthyroid status while receiving hormone substitution. The total removal of the antigenic tissue through total thyroidectomy appears to attenuate the autoimmune response (Chiovato et al., 2003) and ameliorate symptoms (Promberger et al., 2014).

A randomized trial has been conducted (ClinicalTrials.gov: NCT02319538) in 150 patients (with an age of 18–79 years) with persistent HT-associated symptoms even if in euthyroidism while in treatment with L-T4 therapy and with serum AbTPO >1,000 IU/ml (Guldvog et al., 2019). In the follow-up, only the surgical group of patients had an improvement, with an increase in the mean health score from 38 to 64 points, at 18 months. Fatigue score and chronic fatigue frequency decreased. Median circulating AbTPO levels were reduced from 2,232 to 152 IU/mL. Total thyroidectomy ameliorated the quality of life and fatigue in these patients, but not the medical treatment (Guldvog et al., 2019).

CONCLUSION

HT causes a chronic inflammation of the thyroid tissue (McLeod and Cooper, 2012; Antonelli et al., 2015), with a condition of hypothyroidism in ~25% of patients (McLeod and Cooper, 2012; Caturegli et al., 2014; Antonelli et al., 2015; Ragusa et al., 2019), and it was first described with significant signs of AT, lymphocytic infiltration, atrophy of follicular cells, fibrosis, and goiter.

The synthetic hormone L-T4 is recommended as therapy of hypothyroidism-related conditions as it has a chemical structure similar to T4 (Fallahi et al., 2017a).

The tablet formulation of L-T4 needs an acid gastric pH for its absorption (Centanni et al., 2006). In the absence of factors that alter L-T4 absorption, ~70% of tablet L-T4 is absorbed into the duodenum and jejunum (Fallahi et al., 2017a).

Thanks to more sensitive TSH assays, nowadays, the dose of 1.5–1.7 μg/kg body weight is the ideal daily L-T4 replacement dose, able to obtain normal TSH levels in most hypothyroid subjects (Caturegli et al., 2014).

In spite of this, owing to different interfering issues (Virili et al., 2019b), ~20–50% of patients do not respond to the L-T4 treatment (Eligar et al., 2016; Virili et al., 2019b) and require a higher dose (Ernst et al., 2017). Once excluded, a possible pseudomalabsorption linked to a scarce compliance with the prescribed regimen, gastrointestinal disorders, or interfering drugs can cause an altered intestinal absorption of L-T4 and can lead to refractory hypothyroidism (Virili et al., 2019b).

Refractory hypothyroidism and the need to increase the "normal dose" of L-T4 (Centanni et al., 2017; Fallahi et al., 2021) has led to the development of new L-T4 formulations, the oral liquid preparation and soft gel capsule, that have permitted a significant reduction in TSH variability in hypothyroidism, in comparison to tablets.

Moreover, myoinositol has a key role in thyroid autoimmunity and function. A significant decline in TSH and ATA levels has been reported in patients with subclinical hypothyroidism and AT after treatment with myoinositol + selenium, corroborating the immune-modulatory effect of myoinositol (Ferrari et al., 2017b).

Furthermore, thyroidectomy can be rarely recommended in patients with AT. A recent randomized trial suggested that total thyroidectomy can ameliorate quality of life and fatigue in these patients (Guldvog et al., 2019).

In conclusion, in this novel era of precision medicine, further research is needed in larger population to investigate the effect of these new treatments on quality of life.

AUTHOR CONTRIBUTIONS

SMF, SB, AA, and PF conceived the paper. All authors reviewed and approved the final version of the manuscript.

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Biologics in Asthma: A Molecular Perspective to Precision Medicine

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Recent developments in therapeutic strategies have provided alternatives to corticosteroids as the cornerstone treatment for managing airway inflammation in asthma. The past two decades have witnessed a tremendous boost in the development of anti-cytokine monoclonal antibody (mAb) therapies for the management of severe asthma. Novel biologics that target eosinophilic inflammation (or type 2, T2 inflammation) have been the most successful at treating asthma symptoms, though there are a few in the drug development pipeline for treating non-eosinophilic or T2-low asthma. There has been significant improvement in clinical outcomes for asthmatics treated with currently available monoclonal antibodies (mAbs), including anti-immunoglobulin (Ig) E, anti-interleukin (IL)-4 receptor α subunit, anti-IL-5, anti-IL-5Rα, anti-IL-6, anti-IL-33, and anti-thymic stromal lymphopoietin (TSLP). Despite these initiatives in precision medicine for asthma therapy, a significant disease burden remains, as evident from modest reduction of exacerbation rates, i.e., approximately 40–60%. There are numerous studies that highlight predictors of good responses to these biologics, but few have focused on those who fail to respond adequately despite targeted treatment. Phenotyping asthmatics based on blood eosinophils is proving to be inadequate for choosing the right drug for the right patient. It is therefore pertinent to understand the underlying immunology, and perhaps, carry out immune endotyping of patients before prescribing appropriate drugs. This review summarizes the immunology of asthma, the cytokines or receptors currently targeted, the possible mechanisms of sub-optimal responses, and the importance of determining the immune make-up of individual patients prior to prescribing mAb therapy, in the age of precision medicine for asthma.

Keywords: severe asthma, monoclonal antibodies, T2 inflammation, eosinophil, treatment response

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1 INTRODUCTION

Asthma is defined by reversible airflow obstruction, hyperresponsiveness, and inflammation, that manifests as wheeze, dyspnea, and cough. Despite a wide array of treatments available for asthma, 5–10% of patients have poor response to inhaled corticosteroids, and remain on high doses of systemic corticosteroids (Heffler et al., 2019). At first glance, this may seem like a non-significant percentile; however this subgroup contributes substantially to the economical disease burden, accounting for 56 billion US dollars annually, due to frequent exacerbations with need for acute care (Barnett and Nurmagambetov, 2011). Current clinical guidelines for asthma diagnosis include assessment of lung function through spirometry with or without a bronchoprovocation challenge to

quantify hyperresponsiveness. Interestingly, despite airway inflammation being a hallmark feature of asthma, it is not a requirement for asthma diagnosis, but instead helps to stratify disease severity. As a whole, the current tests we have do not account for the vast immunological heterogeneity of asthma.

We have seen great strides over the past 2 decades with respect to the development of alternative therapies to corticosteroids. The era of monoclonal antibodies (mAbs) targeting receptors and cytokines involved in the pathogenesis of asthma has emerged in severe asthma management. Although we have witnessed significant improvement in clinical outcomes for severe asthmatics treated with currently available mAbs, there still remains a proportion of patients with refractory disease. Biologic therapy has used biomarkers to phenotype patients and identify those who would benefit most from therapy, using blood eosinophils, serum total IgE and periostin, and fraction of excreted nitric oxide (FeNO). Unfortunately, these biomarkers fail to reflect the complexity of underlying inflammatory endotypes, and are proving to be inadequate for not only choosing the right drug for the right patient, but also monitoring response to treatment. It has been clear that inflammation in severe asthma is not always characterized by the presence of eosinophilia. We need to pay closer attention to the patients who fail to respond to mAbs to learn lessons on how to better individualize treatment. Immunological endotyping has been proposed as a potential tool to curtail treatment for individual patient's and needs to be further studied. This review will summarize the immunology of asthma, the cytokines or receptors currently targeted, and potential mechanisms of sub-optimal responses.

2 AIRWAY INFLAMMATION IN ASTHMA

Asthma was initially categorized into two simple phenotypes of allergic and non-allergic disease, however over time our understanding of disease pathogenesis has expanded, and we now categorize phenotypes based on underlying inflammatory-based mechanisms (neutrophilic, eosinophilic, mixed, and pauci-granulocytic). There is evidence to suggest that even with each inflammatory phenotype there is a great deal of heterogeneity, with several different immune endotypes contributing to the overlying inflammation. Broadly, there are two asthma endotypes characterized as type 2 (T2) high and T2 low inflammation. The T2-high endotype, defined by a T2 cytokine response (IL-4, IL-5, and IL-13), is the most common endotype and the most well understood.

In order to individualize treatment, a patient's asthma endotype must be identified and fortunately, genomics has emerged as a powerful tool for diagnosis. In severe asthma, three transcriptome-associated clusters (TACs) have been identified, including TAC 1 (*IL-33R*, *CCR3, TSLPR*), TAC2 (*interferon-, tumour necrosis factor alpha-*, and *inflammasome-associated genes*), and TAC3 (*genes of metabolic pathways, ubiquitination and mitochondrial function*). TAC1 has the highest enrichment of gene signatures for IL-13/Th2-high and innate lymphoid cell type 2 (ILC2) inflammation, along with the highest sputum and blood eosinophils and serum periostin. Furthermore, this cluster has oral corticosteroid (OCS)-dependency, frequent exacerbations, and severe airflow obstruction. Conversely, TAC2 has high sputum neutrophils and TAC3 has normal to high sputum eosinophilia and better preserved FEV_1, with the least OCS-dependency. As such, in the setting of severe asthma, three unique clusters of gene expression have been identified, further demonstrating the heterogeneity of endotypes within each inflammatory phenotype.

2.1 Type 2 Inflammation

T2-high inflammation develops in response to cross-talk between innate and adaptive immune responses. Allergic asthma is triggered by inhaled allergens that are taken up within the airways by antigen presenting cells, including dendritic cells. These cells go on to process aeroallergens and present antigen peptides on their cell surface via the HLA class II molecule of the major histocompatibility complex (MHC Class II) within lymph nodes. MHC class II interacts with the T cell receptor (TCR) of naive $CD4^+$ T cells, resulting in polarization towards the T helper 2 (Th2) lineage. Polarization is, in part, driven by IL-4, produced by neighbouring mast cells and basophils.

Once Th2 cells have matured, they migrate to the airways where further antigen exposure results in TCR-antigen binding and prompts Th2 cells to release T2 cytokines including IL-4, IL-5, and IL-13, leading to downstream airway inflammation (Hammad and Lambrecht, 2021). IL-4 and IL-13 induce Ig class switching of B cells to produce IgE, which has the capacity to bind to and activate high-affinity FcεR1 receptors on mast cells and basophils. After initial sensitization, re-exposure to allergen results in IgE crosslinking with FcεR1 receptors, leading to mast cell and basophil degranulation of histamine, leukotrienes, and prostaglandins, which go on to promote bronchoconstriction.

This aforementioned adaptive immune system, is also triggered by upstream innate processes. Inhaled antigens interact with airway epithelium, resulting in production of alarmins including, thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 (Hammad and Lambrecht, 2021). Collectively, these alarmins promote the release of cytokines from Th2 cells, basophils, mast cells, and ILC2s (Salter et al., 2019). Similar to Th2 cells, ILC2s are potent promoters of T2-high inflammation, through production of IL-5 and IL-13 (Salter et al., 2019). In addition, basophils and mast cells, have been identified as potent sources of IL-4 and IL-13 (Bao and Reinhardt, 2015). With respect to IL-13, this cytokine also plays a role in inducing mucus production, airway remodeling, and hyperresponsiveness. In particular, for quite some time the spotlight has been on IL-5, for its an important role in asthma. This eosinophil-maturing cytokine is produced not only by Th2 cells, but also granulocytes and ILC2s. The biologic effects of IL-5 are mediated through interaction with IL-5Rα and a non-specific beta chain heterodimer, recognized by IL-3 and GM-CSF (Rossjohn et al., 2000). When IL-5 is present it binds to IL-5Rα and drives formation of a functional IL-5Rα/β chain receptor complex, that promotes activation of an intricate network of signaling pathways (Johanson et al., 1995).

IL-5Rα is highly expressed on eosinophils (Varricchi et al., 2016) and the interaction between IL-5 and IL-5Rα results in downstream activation of intracellular signaling proteins JAK2 and STAT 1, 3, and 5, which in turn stimulate transcriptional factors involved in eosinophil proliferation (Pazdrak et al., 1995). JAK2 is also involved in the inhibition of eosinophil apoptosis through the active cooperation with Lyn and Raf-1 kinases (Pazdrak et al., 1995; Schwartz et al., 2015). Other signal transduction molecules that are activated by IL-5 include phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK). Through activation of extracellular signal-regulated kinases (ERK)1/2 and protein kinase C (PKC), PI3K mediates IL-5-induced interaction of eosinophils with intracellular adhesion molecule-1 (ICAM-1) (Sano et al., 2005). The Ras-Raf-1-mediated activation of the ERK subfamily of MAPK drives c-fos gene transcription, which is involved in promoting cell maturation, survival, and proliferation (Adachi et al., 2000). Lastly, through a NF-kB-dependent mechanism, p38 MAPK up-regulates eosinophil pro-inflammatory cytokine production (Adachi et al., 2000). IL-5 is responsible for the activation of many integral functions of eosinophils, including maturation, accumulation and activation, and action depends on the interaction with IL-5Rα, mAbs have been developed against IL-5 and IL-5Rα.

Eosinophils exert their effects on the airways through degranulation (principally piecemeal degranulation) including the release of free intact eosinophilic granules (FEGs), which produce tissue-damaging eosinophil granule proteins, including major basic protein (MBP), eosinophilia cationic protein (ECP), eosinophilic-derived neurotoxin (EDN), and eosinophil peroxidase (EPX) (Hogan et al., 2008). Eosinophils also release extracellular DNA deposits that form a web-like structure called eosinophilic extracellular traps (EETs) through a process called ETosis (Mukherjee et al., 2018a). EETs have an autocrine effect on promoting eosinophil degranulation and inducing epithelial cells to produce IL-6 and IL-8 (Mukherjee et al., 2018a). Collectively, the aforementioned mediators contribute to airway remodeling, airway hyperreactivity, and increased mucus production.

2.2 Type 1 Inflammation

T2-low inflammation has emerged as another pathway that results in asthma pathogenesis. Pattern recognition receptors (PRRs) on surface of airway epithelial cells, granulocytes, dendritic cells, and T cells, act to recognize danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) and induce downstream mediator release. Activation of the PRR called nucleotide-binding oligomerization domain-like receptor (NLR) results in stimulation of inflammasomes, which are multimolecular signaling platforms that act as critical inducers of host defense (Elliott and Sutterwala, 2015). In particular, the NLRP3 inflammasome is one of the five major inflammosomes that induces secretion of IL-1β (Elliott and Sutterwala, 2015). This secretion is mediated through caspase-1, which cleaves IL-1β into its secretory isoform. IL-1β plays a role in Th17 differentiation and IL-17 production. IL-17 acts as an important mediator of neutrophilic inflammation and is elevated in severe asthmatics with frequent exacerbations (Ricciardolo et al., 2017). The inflammasome promotes pyroptosis, a form of lytic cell death. NLRP3, caspase-1, and IL-1β are increased in sputa of severe asthmatics and correlate with disease severity (Simpson et al., 2014; Kim et al., 2017). Neutrophils have been proposed to play a role in activation of the inflammasome (Wright et al., 2016). Neutrophil-derived extracellular DNA (eDNA) is released in a web-like structure to form neutrophil extracellular traps (NETs), in a process known as NETosis, which can be induced by infectious and non-infectious conditions. The presence of airway neutrophilia and NETosis results in inflammasome activation, leading to promotion of Th17-mediated inflammation. Although there are emerging biologic therapies that target T2-low inflammation, the overall identification of this endotype and use of biomarkers to monitor treatment response remains largely unknown.

3 CURRENTLY AVAILABLE BIOLOGICAL THERAPY AND SUBOPTIMAL RESPONSES IN SEVERE ASTHMA

The practice of precision medicine in asthma is far from optimal due to lack of complete understanding of the complex immunological nature of asthma. The severe asthma group is quite heterogeneous in nature and as such, a "one size fits all" approach cannot be used to manage these patients. Although many severe asthmatics have T2-high inflammation, the underlying mechanisms driving this inflammation may vary drastically across patients. This problem has been underscored by the high degree of variation in patient response to biologic therapy, where some patients respond dramatically and others either fail treatment or have suboptimal responses. Super-responders (SR) are defined as having improvement across three or more domains over a 12-month period including exacerbation elimination and improvement in asthma control (Upham et al., 2021). A better understanding is needed on how to identify these SR and determine what characteristics predispose them to a dramatic response to biologic therapy. Similarly, we need to better identify treatment failures and suboptimal responders to determine what underlying mechanisms contribute to this and how they differ from SR. In this section, we will review evidence behind current biologics and potential underlying mechanisms accountable for suboptimal response and treatment failure. The targeted pathways along with the key studies pertaining to these biologics are summarized in **Figure 1** and **Tables 1**–**7** respectively, while **Figure 2** summarises the possible factors associated/contributing to poor therapeutic responses.

3.1 IgE Targeted Therapy

IgE is the primary immunoglobulin involved in T2-high inflammation. Omalizumab binds to the third constant region of IgE and prevents free IgE from interacting with high and low-affinity FcεR1 receptors (Fahy et al., 1997). As a result of this binding, free serum IgE levels decrease, as well as the overall IgE receptor density on mast cells and basophils. Numerous

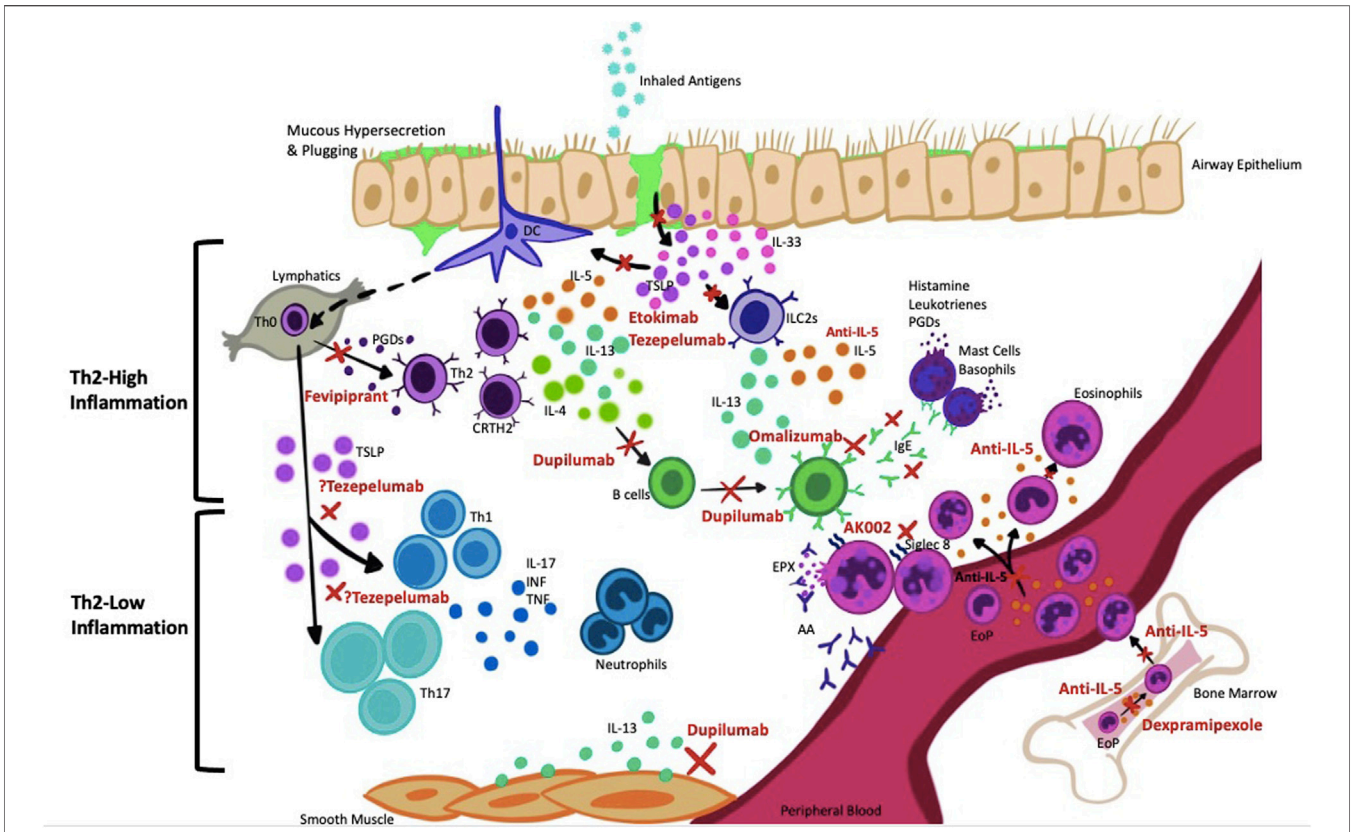

FIGURE 1 | Targets of current therapy in severe asthma. There are numerous targets that have been identified for the management of severe asthma. In particular, the differentiation of eosinophil progenitors (EoP) into eosinophils, and subsequent activation of eosinophils can be targeted by anti-Siglec-8 (AK002), dexpramipexole, as well as anti-IL-5 therapies. The secretion of alarmin cytokines (TSLP, IL-33) from epithelial cells and activation of downstream ILC2s and Th2 cells can be inhibited by anti-TSLP and anti-IL-33 agents, such as tezepelumab and etokimab, respectively. The downstream action of Th2 cytokines, such as IL-4 and IL-13, produced primarily from Th2 cells, ILC2s and basophils can be inhibited by dupilumab. The cross-linking of IgE on FcεR1 receptors on mast cells and basophils can be inhibited by omalizumab, and thus prevent degranulation of leukotrienes, histamine, and prostaglandins (PGDs). PGDs play an important role in binding to CRTH2 on ILC2s and Th2 cells, promoting their migration and activation within the airways. This can be targeted by anti-CRTH2 agents such as fevipiprant. There are few identified targets for Th2-low inflammation but anti-TSLP is a potential biologic acts on this pathway. Abbreviations: AA: Autoantibodies; EoP: Eosinophil Progenitors; ILC2s: Innate Lymphoid Type 2 Cells; INF: Interferon; PGDs: Prostaglandins; TNF: Tumor necrosis factor; TSLP: Thymic Stromal Lymphophoietin.

randomized clinical trials (RCTs) and real-life studies have shown that treatment of asthmatics with omalizumab results in a dose-dependent reduction in free IgE in serum, improvement in lung function, and modest reduction in exacerbation rates, as well as emergency visits and hospitalizations (Corne et al., 1997; Hanania et al., 2011; Rodrigo et al., 2011; Normansell et al., 2014). RCTs have also shown improvement in symptom control, quality of life, and reduced oral corticosteroid (OCS) use (**Table 1**) (Humbert et al., 2005; Rodrigo et al., 2011; Normansell et al., 2014; Pelaia et al., 2018a). In terms of molecular findings, omalizumab reduces both eosinophil and basophil infiltration within the airways (Rodrigo et al., 2011; Normansell et al., 2014; Pelaia et al., 2018a). Of note, a large retrospective analysis of 25 RCTs demonstrated greater reduction in asthma exacerbation patients who specifically had high blood eosinophilia and fractional exhaled nitric oxide (FeNO) levels, which was suggestive of eosinophilic inflammation (Hanania et al., 2013). Based on these findings, it seemed justified to prescribe anti-IgE biologics to severe asthmatics with evidence of atopy. As promising as the aforementioned studies are, they were primarily based on mild-moderate asthmatics, and more studies are needed to determine efficacy of these agents in severe corticosteroid-dependent asthma.

3.1.1 Possible Reasons for Suboptimal Responses With Anti-IgE

Agents that target IgE-dependent mechanisms have been shown to be efficacious in mild to moderate asthma, however, this pathway may not be the major driver of eosinophilic inflammation in severe asthma. Thus, although anti-IgE agents may sufficiently suppress IgE-dependent mechanisms, the IgE-independent pathways are still active within the airways, continuing to drive Th2 inflammation. This is, in part, supported by studies showing that omalizumab treatment in severe asthma does not reduce sputum eosinophils, (Mukherjee et al., 2019) and that pediatric patients with severe asthma are low responders to omalizumab (Garcia et al., 2013). Chapman et al. (2019) assessed the efficacy of using mepolizumab

TABLE 1 | Summary of anti-IgE targeted randomized clinical trials in severe asthma.

Anti-IgE						
Landmark study and year	**Study type**	**Asthma severity**	**Asthma phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
Hanania et al. (2013)	Phase 2	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q2W, Q4W Route: SC Duration: 48 W	-Reduced AAER -Improved AQLQ, FEV_1	-Reduced FeNO
INNOVATE, 2004 Busse et al. (2019)	Phase 2	Severe uncontrolled asthma	Atopic	Dose: 0.008–0.016 mg/kg IgE (IU/ml) Frequency: Q2W, Q4W Route: SC Duration: 28 W	-Reduced AAER, ED visits -Improved morning PEF	-N/A
Busse et al. (2001)	Phase 3	Severe uncontrolled asthma	Atopic	Dose: 0.008–0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 28 W	-Reduced AAER, steroid dose -Improved morning PEF, ACQ	-Reduced serum IgE
Garcia et al. (2013)	Phase 3b	Severe uncontrolled asthma	Atopic	Dose: 200, 300 mg Frequency: Q2W Route: SC Duration: 16 W	-No change in ACQ or AAER -Increased FEV_1	-FcεR1 decreased on basophils and DCs at 16 W
Djukanović et al. (2004)	Phase 3	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 16 W	-No improvement in AHR	-Reduced SP, submucosal and epithelial eosinophils -Reduced FcεR1+ and IgE + cells, CD4/CD3/CD8 T cells, and IL-4+ cells in submucosa -Reduced serum IgE
Chanez et al. (2010)	Phase 3	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 16 W	-No change in AAER	-Reduced FcεR1 on basophils and DCs at 16 W
de Llano et al. (2013)	Phase 3	Severe uncontrolled asthma	Atopic and non-atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 24 M	-Improved GETE scale and ACT score -Increased FEV_1 -No change in AAER	-N/A
XCLUSIVE, 2011 Schumann et al. (2012)	Phase 3	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 6 M	-Increased FEV_1 at 16 W -Improved ACQ at 16 W -Reduced AAER	-N/A
Holgate et al. (2005)	Phase 3	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 16 W	-Reduced fluticasone dose	-N/A
Barnes et al. (2013)	Retrospective study	Severe uncontrolled asthma	Atopic	Dose: 0.016 mg/kg IgE (IU/ml) Frequency: Q4W Route: SC Duration: 12 M	-Reduced steroid use/dose, hospitalizations and ED visits, and AAER -Increased FEV_1	-N/A

AAER: annualized asthma exacerbation ratio; ACQ, asthma control questionnaire; AHR: airway hyperresponsiveness; ACT, asthma control test; AQLQ, asthma quality of life questionnaire; DC, dendritic cell; ED, emergency department; FeNO, fraction of expired nitric oxide; GETE, global evaluation of treatment effectiveness; IU, international units; M, months; NA, not applicable; Q, every; SC, subcutaneous; W, weeks.

TABLE 2 | Summary of randomized clinical trials assessing mepolizumab in severe asthma.

Anti-IL-5: mepolizumab

Landmark study and year	RCT phase	Asthma severity	Asthma phenotype	Dosing, duration and route of administration	Clinical effect	Molecular effect
Nair et al. (2009)	Phase 2	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 750 mg Route: IV Frequency: Q4W Duration: 26 W	-Reduced AAER and time-to-exacerbation -Reduction in prednisone dose -Improved FEV_1 and ACQ	-No exacerbations associated with SP eosinophilia, instead there was SP neutrophilia -Reduced SP and PB eosinophils
Haldar et al. (2009)		Severe uncontrolled asthma	≥3% sputum eosinophils in previous 2 years	Dose: 750 mg Route: IV Frequency: Q4W Duration: 52 W	-57% reduction in AAER at 50 W -Improved AQLQ score -No change in FEV_1 post-BD use or AHR	-Reduced PB and SP eosinophils -No change in FeNO or neutrophil count in SP
DREAM, 2012 Pavord et al. (2012); Ortega et al. (2016)	Phase 2	Severe uncontrolled asthma	≥0.3×10^9 cells/L PB eosinophils or FeNO ≥50 ppb or SP eosinophils ≥3%	Dose: 75–750 mg Route: IV Frequency: Q4W Duration: 52 W	-48% reduction in exacerbations at 52 W -60% reduction in exacerbations requiring hospitalization or ED visits -No difference in AQLQ, ACQ sores or FEV_1	-Reduced PB and SP eosinophils
MENSA, 2014 Ortega et al. (2014); Ortega et al. (2016)	Phase 3	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 75, 100 mg Route: IV, SC Frequency: Q4W Duration: 32 W	-53% reduction in AAER at 32 W -61% reduction in ED visits or hospitalizations at 32 W -Improved FEV_1, PEF, SGRQ and ACQ ($p < 0.05$)	-Reduced PB eosinophils by 4 W
MUSCA, 2017 Chupp et al. (2017)	Phase 3b	Severe uncontrolled asthma	≥0.3×10^9 cells/L PB eosinophils or FeNO ≥50 ppb or SP eosinophils ≥3%	Dose: 100 mg Route: IV Frequency: Q4W Duration: 52 W	-58% reduction in AAER at 24 W -68% reduction in hospitalizations and ED visits at 24 W -Improvement in SQRQ score at 4 W -Improved pre-BD FEV_1 at 24 W	-N/A
SIRIUS, 2017 Bel et al. (2014)	Phase 3	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: IV Frequency: Q4W Duration: 52 W	-32% reduction in AAER 24 W -50% reduction in OCS dose at 24 W -Improved ACQ and SQRQ at 24 W - No change in FEV_1 at baseline or post-BD	-N/A
COSMOS, 2016 Lugogo et al. (2016)	Phase 3	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: SC Frequency: Q4W Duration: 52 W	-Maintained reduced exacerbation rates and OCS dosing	-N/A

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TABLE 2 | (*Continued*) Summary of randomized clinical trials assessing mepolizumab in severe asthma.

Anti-IL-5: mepolizumab						
Landmark study and year	**RCT phase**	**Asthma severity**	**Asthma phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
COLUMBIA, 2019 Khatri et al. (2019)	Phase 3	Severe uncontrolled asthma	≥300 cells/uL PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: SC Frequency: Q4W Duration: 52 W	-61% reduction in AAER -Improved ACQ-5 at 24 W -Improved pre-BD FEV_1 at 24 W	-N/A
COSMEX, 2019 Khurana et al. (2019)	Phase 3b	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: SC Frequency: Q4W Duration: 172 W	-Maintained reduced AAER and daily OCS use -Improved FEV_1 and ACQ-5	-Reduced PB eosinophils
OSMO, 2019 Chapman et al. (2019)	Phase 4	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: SC Frequency: Q4W Duration: 32 W	-64% reduction in AAER at 32 W -69% reduction in hospitalizations and ED visits at 32 W -Improved SGRQ and pre-BD FEV_1 at 32 W	-Reduced blood eosinophils, ECP, EDN at 32 W
Bagnasco et al. (2018)	Prospective cohort study	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	12 M post-initiation of mepolizumab	-Reduction in OCS-dependence and exacerbation rate	-N/A
REALITI-A, 2020 Harrison et al. (2020)	Prospective cohort study	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	12 M post-initiation of mepolizumab	-Reduced AAER, hospitalizations and ED visits -Reduced OCS maintenance dose	-Reduced PB eosinophils
Pelaia et al. (2018a); Pelaia et al. (2018b)	Single-centered observational study	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils in previous year or ≥150 cells/μl PB eosinophils at screening	Dose: 100 mg Route: SC Frequency: Q4W Duration: 24 W	-Increased ACT score after 24 W -Improved FEV_1 and FEV_1/FVC after 24 W -Reduced exacerbation frequency -Decreased prednisone dose	-Reduced PB eosinophils at 24 W

to treat severe eosinophilic asthmatics who were inadequately controlled on omalizumab. Interestingly, subgroup analysis demonstrated no additional benefit when both biologics were in the system, nor was there a decline seen in the benefit of omalizumab as it washed out. However, the patients who showed the most improvement in asthma control were those with eosinophilia (≥150 cells/μl blood eosinophils). These findings suggest that singular targeting of IgE-dependent mechanisms may not be effective in all inflammatory subtypes of severe asthma and that perhaps targeting IgE is more beneficial in patients with underlying atopic status. In addition, there have been reports of IgG autoantibodies generated against IgE in allergic asthma and the formation of IgE-IgG heterocomplexes in autoimmune conditions that trigger innate immune cells (Chan et al., 2014; Henault et al., 2016). Thus, the presence of autoantibodies and immune complexes in allergic airways may impede the action of anti-IgE mAb and inadvertently induce a continued need for OCS. More studies are needed to determine what particular inflammatory profiles of severe asthma would benefit most from IgE blockade with omalizumab and if its combination with agents that target IgE-independent mechanisms would provide a synergistic effect.

3.2 IL-5 Targeted Therapy

Given the role of IL-5 in driving eosinophilic inflammation, it was proposed that blockade of this cytokine may attenuate T2-high inflammation. There have been three agents developed so far that target IL-5. Mepolizumab and reslizumab bind to IL-5, thereby preventing this cytokine from promoting eosinophil activation. Benralizumab, alternatively blocks the IL-5Rα, resulting in near

TABLE 3 | Summary of randomized clinical trials assessing reslizumab in severe asthma.

Anti-IL-5: reslizumab

Landmark study and year	Study format	Asthma severity	Asthma phenotype	Dosing, duration and route of administration	Clinical effect	Molecular effect
Castro et al. (2011)	Phase 2	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 15 W	-Improved FEV_1 but no change in ACQ or AAER	-Reduced SP eosinophils
Castro et al. (2015)	Phase 3	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 52 W	-Reduced AAER	-N/A
Bjermer et al. (2016)	Phase 3	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 16 W	-Improved ACQ, AQLQ, FEV_1 and FVC	-N/A
Corren et al. (2016)	Phase 3	Severe uncontrolled asthma	≥400 or <400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 16 W	-No change in mean FEV_1, except in subgroup analysis with eosinophilia	-Reduced PB eosinophils
Murphy et al. (2017)	Phase 3	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 24 M	-Improved ACQ, AQLQ, FEV_1 and FVC	-Reduced PB eosinophils
Brusselle et al. (2017)	Phase 3	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 52 W	-Reduced AAER over 52W, and exacerbations requiring hospitalization/ED visits -Improved ACQ and AQLQ for late onset patients	-N/A
Weinstein et al. (2019)	Phase 3	Severe uncontrolled asthma with CRS	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 52 W	-Reduced AAER -Improved FEV_1	-N/A
Bernstein et al. (2020)	Phase 3	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils	Dose: 110 mg Route: SC Frequency: Q4W Duration: 52 W	-No difference in AAER, except in those with PB eosinophils ≥400 -No difference in steroid dosing	-N/A

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TABLE 3 | (*Continued*) Summary of randomized clinical trials assessing reslizumab in severe asthma.

Anti-IL-5: reslizumab						
Landmark study and year	**Study format**	**Asthma severity**	**Asthma phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
Mukherjee et al. (2018b)	Placebo-Controlled Sequential Trial	Severe uncontrolled asthma previously on 1Y of mepolizumab	≥3% SP and ≥300 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 12 W	-Improved FEV_1 and ACQ	-Reduced SP and PB eosinophils, SP EPX, anti-EPX, and ANA -Reduced PB HPC, EoP, and SP $CD4^+$ T cells, no change in ILC2 in PB or SP
Ibrahim et al. (2019)	Prospective Cohort	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 2 Y	-Improved ACQ at baseline and up to 2Y -Reduced OCS dose and/or use at 1Y -Reduced AAER at 1Y	-Reduced PB eosinophils
Pérez de Llano et al. (2019)	Open-label Prospective Study	Severe uncontrolled asthma who failed omalizumab	≥400 cells/μl PB eosinophils	Dose: 3 mg/kg Route: IV Frequency: Q4W Duration: 24 W	-Improved ACT score at 4, 12 W -Improved ACQ score at 12, 24 W -60% of patients controlled at 24 W	-Reduced PB eosinophils and FeNO at 24 W

complete depletion of peripheral eosinophils through antibody-dependent cell-mediated cytotoxicity (ADCC) involving NK cells.

The story of mepolizumab provides a valuable lesson for individualized asthma management. Leckie et al. first showed in 2000 that despite mepolizumab resulting in attenuation of blood and sputum eosinophils, there was a lack of translation into meaningful clinical outcomes in patients with asthma (Leckie et al., 2000). Large RCTs later followed, which also failed to show clinical efficacy, and as a result the development of anti-IL-5, or indeed any anti-eosinophil therapy, was tabled for many years (Flood-Page et al., 2003). In fact, the overall importance of IL-5 and eosinophils in asthma was brought into question. It should be noted that these initial trials did not select appropriate patient populations based on eosinophilia and a T2-high profile, but rather selected a heterogeneous pool of asthmatic patients with a variety of immunological profiles. Hence, it is not surprising that there was a lack of clinical response to anti-IL-5 treatment in these early studies.

This led to a pivotal change in studies examining anti-IL-5 therapy through the specific targeting of T2-high patients. Using patient selection criteria for the T2-high profile, Haldar et al. reported that mepolizumab treatment in severe asthmatics that specifically exhibited eosinophilia (≥3% sputum eosinophils in last 12 months), not only reduced blood and sputum eosinophils but also resulted in 43% fewer exacerbations (Haldar et al., 2009). Further, Nair et al. showed that mepolizumab administration to severe prednisone-dependent eosinophilic asthmatics resulted in OCS tapering, where patients had an 83% reduction of their maximum prednisone dose versus 47% with placebo. They also reported fewer exacerbations, improved asthma control, and increased FEV_1 with an associated decrease in sputum and blood eosinophils (Nair et al., 2009). Multiple other RCTs and real-world investigations have shown that mepolizumab has a corticosteroid-sparing effect in this population, with reductions in annual asthma exacerbation rates (AAER) by 39–52%, improvement in lung function, and asthma symptom scores, as well as improvement in overall health-related quality of life (**Table 2**) (Pavord et al., 2012; Bel et al., 2014; Chupp et al., 2017; Pelaia et al., 2018b; Harrison et al., 2020).

Three important observations should be noted from the mepolizumab data. Firstly, the efficacy of mepolizumab is based on patients having baseline eosinophilia, and as such, we must identify individuals that will benefit from this treatment in the first place. Peripheral blood eosinophils were initially chosen as a biomarker in the mepolizumab studies for a few reasons. Peripheral eosinophils have been identified through cluster analyses to predict responsiveness to mepolizumab (Ortega et al., 2016). Indeed, it is one of the most simple and practical ways to identify Th2 inflammation and as such, patients that may benefit from anti-IL-5 therapy. The caveat to this is that peripheral eosinophils can be highly variable and a single measurement of peripheral blood eosinophils may not reflect the average level of cells throughout an extended period of time. Thus, the use of peripheral blood eosinophils to guide therapy to anti-IL-5 biologics may not be as ideal as sputum eosinophils.

TABLE 4 | Summary of randomized clinical trials assessing benralizumab in severe asthma.

Anti-IL-5R alpha: benralizumab

Landmark study and year	Study format	Asthma severity	Asthma phenotype	Dosing, duration and route of administration	Clinical effect	Molecular effect
Nowak et al. (2015)	Phase 2	Severe uncontrolled asthma	<450 cells/μl or ≥450 cells/μl PB eosinophils	Dose: 0.3 mg/kg or 1 mg/kg Route: SC Frequency: Once Duration: 24 W	-49% reduction in AAER -60% reduction in hospitalization -No change in FEV_1, ACQ, AQLQ	-Reduced PB eosinophils up to 12 W -Reduced ECP and EDN
CALIMA, 2016 FitzGerald et al. (2016)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 56 W	-28% reduction in AAER at 56 W -Improved ACQ, AQLQ, pre-BD FEV_1	-Reduced PB eosinophils
SIROCCO, 2016 Bleecker et al. (2016)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 48 W	-Reduced AAER, regardless of eosinophils at 48 W -Improved FEV_1, ACQ-6, AQLQ at 48 W	-Reduced PB eosinophils by 4 W
ZONDA, 2017 Nair et al. (2017)	Phase 3	Severe uncontrolled asthma	≥150 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 28 W	-75% reduction in OCS dose -Improved AAER, ACQ-6, AQLQ -No effect on FEV_1	-N/A
BISE, 2017 Ferguson et al. (2017)	Phase 3	Mild-moderate persistent asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W Duration: 12 W	-Increased pre-BD FEV_1 at 12 W	-N/A
Chupp et al. (2019)	Phase 3	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 28–56 W	-Improvement in morning PEF from baseline within 2 W	-N/A
BORA, 2019 Busse et al. (2019)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W Q8W Duration: 56 W	-72% of patients with eosinophilia did not have exacerbation -Maintained improvement in FEV_1 and ACQ, AQLQ	-N/A

(Continued on following page)

TABLE 4 | (*Continued*) Summary of randomized clinical trials assessing benralizumab in severe asthma.

Anti-IL-5R alpha: benralizumab						
Landmark study and year	**Study format**	**Asthma severity**	**Asthma phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
Gauvreau et al. (2021)	RCT	Mild asthma	Atopic	Dose: 30 mg Route: SC Frequency: Q4W Duration: 12 W	-N/A	-Reduced SP eosinophils at 7 h post-allergen challenge -Reduced PB, BM and SP before and 24 h post-allergen challenge -Incomplete depletion of basophils in PB and BM pre- and post-24 h allergen in challenge, no effect on SP basophils
Sehmi et al. (2018)	RCT	Severe uncontrolled asthma	≥3% sputum eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 28 W	-N/A	-Reduced BP and SP eosinophils -Reduced PB EoP -Reduced IL-5-stimulated Eo/B CFU
J-BEST, 2019 Izumo et al. (2020)	Prospective	Severe uncontrolled asthma	≥300 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W, Q8W Duration: 4–12 W	-Improved FEV_1, ACT, AQLQ	-Decreased PB eosinophils and basophils, but no change in FeNO or serum total IgE
Kavanagh et al. (2021)	Prospective	Severe uncontrolled asthma	≥400 cells/μl PB eosinophils	Dose: 30 mg Route: SC Frequency: Q4W Q8W Duration: 48 W	-Reduced AAER -Improved FEV_1, ACQ, AQLQ	-Reduced PB eosinophils -No change in FeNO
PONENTE, 2019 (ongoing) Menzies-Gow et al. (2019)	Phase 3b	Severe uncontrolled asthma	≥150 cells/μl PB eosinophils at enrollment or ≥300 cells/μl PB eosinophils in last 12 M	Dose: 30 mg Route: SC Frequency: Q4W Q8W Duration: 32 W	Pending	Pending

Unfortunately, there is a lack of global availability of sputum labs making it difficult to use sputum eosinophils as a universal biomarker. However, if available, sputum counts can be reproducible and reliable if the proper processing technique is done. We propose that peripheral eosinophilia may be helpful to predict response to anti-IL-5 agents but has a limited role in monitoring response to treatment. As previously reported, in 250 patients with baseline blood eosinophilia (≥400 cells/μl) treated with either mepolizumab or reslizumab for at least 4 months, there was an overall suboptimal response in 43% (Mukherjee et al., 2018b). Of the 129 patients in whom paired blood and sputum eosinophils were available 4 months post-treatment, there were 65 suboptimal responders, 78% of who had sputum eosinophils ≥3%. Only seven of these patients had blood eosinophils ≥400 cells/μl. As such, there is a discordance between the two compartments, which may be, in part, due to *in situ* eosinophilopoiesis. As such, the use of sputum eosinophils to monitor response to treatment may be more reliable than peripheral eosinophils. Secondly, despite multiple studies showing clinical benefit, the effect of mepolizumab was ultimately incomplete, with ~50% reduction in exacerbation rates despite ablation of peripheral eosinophilia. This begs the question as to whether peripheral blood eosinophils should be the only biomarker to determine whether patients would benefit from this biologic. Lastly, and most importantly, we need to re-examine the spotlight on eosinophils and their overall importance in T2-high inflammation. Aside from eosinophils, there are cytokines and effector cells, which may be equally, if not more important than eosinophils, and as such, should be targeted.

Reslizumab was the second anti-IL-5 biologic to be brought to market. In contrast to mepolizumab, it is administered intravenously with weight-based dosing. A phase 2 RCT showed that reslizumab reduced both sputum and blood eosinophils, with an associated improvement in FEV_1 (Kips

TABLE 5 | Summary of randomized clinical trials assessing IL-4/13 in severe asthma.

Anti-IL-4/13

Landmark study and year	RCT phase	Asthma severity	Inflammatory profile	Dosing, duration and route of administration	Clinical effect	Molecular effect
Hodsman et al. (2013)	Phase 1	Mild asthma	Independent of PB eosinophils	Biologic: GSK679586 Dosing: 0.005–10 mg/kg Frequency: Q4W Route: IV Duration: 28 W	-N/A	-Increased serum IL-13 -Reduced FeNO at 2W and 8 W
De Boever et al. (2014)	Phase 2	Severe uncontrolled asthma	≥140 cells ul PB eosinophils	Biologic: GSK679586 Dosing: 10 mg/kg Frequency: Q4W Route: IV Duration: 24 W	-No change in ACQ score, FEV_1, AAER	-No difference in serum IL-13 or IgE -No difference in PB eosinophils
Piper et al. (2013)	Phase 2a	Moderate-severe uncontrolled asthma	Independent of PB eosinophils	Biologic: Tralokinumab Dosing: 150–600 mg Frequency: Q2W Route: SC Duration: 24 W	-No change in ACQ, pre-BD FEV_1, FVC, PEF, AAER.	N/A
Russell et al. (2018)	Phase 2	Moderate-severe uncontrolled asthma	Independent of PB eosinophils	Biologic: Tralokinumab Dosing: 300 mg Frequency: Q2W Route: SC Duration: 12 W	-N/A	-No change in bronchial eosinophils at 12 W - No change in PB or SP eosinophils or serum IgE
STRATOS I, II, 2018; Panettieri et al. (2018)	Phase 3	Severe uncontrolled asthma	≥37 ppb FeNO or <37 ppb	Biologic: Tralokinumab Dosing: 300 mg Frequency: Q2W, Q4W Route: SC Duration: 52 W	-Reduced AAER at 2 W in FeNO-high patients	-N/A
Corren et al. (2011)	Phase 2	Severe uncontrolled asthma	Periostin ≥50 or <50 ng/ml	Biologic: Lebrikizumab Dose: 250 mg Frequency: Q4W Route: SC Duration: 24 W	-60% reduction in exacerbation at 24 W -Improved FEV_1, at 12 W -No change in ACQ	-19% reduction in FeNO at 12 W -Decreased CCL13, CCL17, total IgE levels at 24 W
Noonan et al. (2013)	Phase 2	Mild asthma	Periostin ≥50 or <50 ng/ml	Biologic: Lebrikizumab Dose: 125–500 mg Frequency: Q4W Route: SC Duration: 12 W	-No change in FEV_1, pre-PB PEF, AQLQ	-N/A
LUTE, VERSE, 2015 Hanania et al. (2015)	Phase 3	Severe uncontrolled asthma	Periostin ≥50 or <50 ng/ml	Biologic: Lebrikizumab Dose: 37.5–250 mg Frequency: Q4W Route: SC Duration: 52 W	-60% reduction in exacerbation in periostin-high patients -No dose response for exacerbation -Improved FEV_1 at 12 W	- Reduction in PB eosinophils and FeNO

(Continued on following page)

TABLE 5 | (*Continued*) Summary of randomized clinical trials assessing IL-4/13 in severe asthma.

Anti-IL-4/13

Landmark study and year	RCT phase	Asthma severity	Inflammatory profile	Dosing, duration and route of administration	Clinical effect	Molecular effect
LAVOLTA I, II, 2016; Hanania et al. (2016)	Phase 2	Severe uncontrolled asthma	≥300 cells μl PB eosinophils or periostin ≥50 ng/ml	Biologic: Lebrikizumab Dose: 37.5–125 mg Frequency: Q4W Route: SC Duration: 52 W	-70% reduction in exacerbation in periostin-high patients	-N/A
STRETTO, 2018; Korenblat et al. (2018)	Phase 3	Mild-moderate asthma	≥300 cells μl PB eosinophils or periostin ≥50 ng/ml	Biologic: Lebrikizumab Dose: 125 mg Frequency: Q4W Route: SC Duration: 12 W	-No change in FEV_1, pre-PB PEF, AQLQ	-N/A
Wenzel et al. (2007)	Phase 2a	Atopic asthma	Independent of PB eosinophils	Biologic: Pitrakinra Dose: 25, 60 mg Frequency: OD, BID Route: SC, Inhaled Duration: 12 W	-No change in FEV_1 for SC trial but reduction with inhaled	- Decreased FeNO with inhaled group - No change in SP or PB eosinophils - No change in serum IgE
Otulana et al. (2011)	Phase 2b	Moderate-severe uncontrolled asthma	Independent of blood eosinophils or atopic status	Biologic: Pitrakinra Dose: 1–10 mg BID Route: Inhaled Duration: 12 W	-Reduced exacerbation in eosinophilic group -Improvement in symptom scores and spirometry	-N/A
Corren et al. (2010)	Phase 2	Moderate-severe asthma	Independent of blood eosinophils or atopic status	Biologic: AMG 317 Dose: 75–300 mg Frequency: Q4W Route: SC Duration: 12 W	-No improvement in ACQ -No decrease in exacerbation	- No change in serum IgE - No change in sputum eosinophils, FeNO
Wenzel et al. (2013)	Phase 2	Moderate-severe asthma	≥300 cells μl PB eosinophils or SP eosinophils ≥3%	Biologic: Dupilumab Dose: 300 mg Frequency: Q1W Route: SC Duration: 12 W	-87% reduction in exacerbation at 12 W -Increase in FEV_1 predicted from 2 to 12 W -Improved ACQ at 3 W	- Reduced FeNO from 4 to 12 W - Decrease in serum TARC, eotaxin-3 or IgE - No change in PB or SP eosinophils
Wenzel et al. (2016)	Phase 2b	Severe uncontrolled asthma	<300 cells/ul or ≥300 cells/μl PB eosinophils	Biologic: Dupilumab Dose: 200, 300 mg Frequency: Q2-4W Route: SC Duration: 24 W	-Increased FEV_1 in those with PB ≥ 300 cells/uL eosinophils -Reduced AAER -Improved ACQ, regardless of eosinophils	-Reduced FeNO at 24 W
LIBERTY ASTHMA QUEST, 2018; Castro et al. (2018)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Biologic: Dupilumab Dose: 200, 300 mg Frequency: Q2W Route: SC Duration: 52 W	-Reduced AAER -Increased FEV_1 at 12 W -Improved ACQ, AQLQ scores	-Reduced FeNO, serum IgE, periostin, eotaxin-3, TARC at 52 W -Transient increased PB eosinophils with increased ECP

(Continued on following page)

TABLE 5 | (*Continued*) Summary of randomized clinical trials assessing IL-4/13 in severe asthma.

Anti-IL-4/13						
Landmark study and year	**RCT phase**	**Asthma severity**	**Inflammatory profile**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
LIBERTY ASTHMA QUEST, 2018; Castro et al. (2018)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Biologic: Dupilumab Dose: 300 mg Frequency: Q2W Route: SC Duration: 24 W	-Reduced OCS dose -Reduced rate of severe asthma exacerbations and AAER -Increased FEV_1 -Improved ACQ	-Reduced FeNO -Transient increased PB eosinophils
LIBERTY ASTHMA VENTURE, 2020; Rabe et al. (2020)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells/μl PB eosinophils	Biologic: Dupilumab Dose: 300 mg Frequency: Q2W Route: SC Duration: 24 W	-Improved pre-BD FEV_1, FVC, FEV_1/FVC - Reduced AAER	-N/A
Maspero et al. (2020)	Phase 3	Moderate-severe uncontrolled asthma and CRS	≥150 cells/μl or ≥300 cells/μl PB eosinophils	Biologic: Dupilumab Dose: 200, 300 mg Frequency: Q2W Route: SC Duration: 24 W	-Increased pre- and post-BD FEV_1 in CRS and non-CRS groups -Improved ACQ, AQLQ, SNOTT-22 scores in CRS and non-CSR groups	-Decrease in FeNO, serum IgE and TARC in CRS and non-CRS groups -No change in PB eosinophils in non-CRS group, but mild elevation in CRS group

et al., 2003). Subsequent RCTs in severe asthmatics with blood eosinophils ≥400 cells/μl, have shown that reslizumab reduces AAER by 50–60%, improves symptom scores and lung function, and reduces blood eosinophils (**Table 3**) (Castro et al., 2011; Castro et al., 2015; Bjermer et al., 2016; Corren et al., 2016; Brusselle et al., 2017; Máspero, 2017; Murphy et al., 2017; Bernstein et al., 2020). Finally, post-hoc analysis suggested that patients who do not respond to the fixed-dose regimen of mepolizumab may benefit from reslizumab as an alternative (Mukherjee et al., 2018b). Similar to mepolizumab, reslizumab seems to have the most evidence for clinical efficacy in those with peripheral eosinophilia, however, the reduction in exacerbation rates remains incomplete.

Benralizumab, an antibody to the alpha subunit of the IL-5 receptor, was the third IL-5 pathway-targeting biologic that came to fruition. Phase 3 RCTs have shown that severe eosinophilic asthmatics (≥150 cells/μl blood eosinophils in last 12 months) treated with benralizumab 30 mg SC q4 weeks, were able to reduce their prednisone dosing, exhibited reduced AAER by 28–55%, and also showed improved lung function and symptom scores. From a cellular and molecular standpoint, benralizumab reduced peripheral and sputum eosinophils, along with diminished eosinophil products, including ECP and EDN (Nowak et al., 2015; FitzGerald et al., 2016; Sehmi et al., 2018). Expanding on this, benralizumab can also deplete basophils within peripheral blood in uncontrolled asthma (Eck et al., 2014), however a new study has shown that in mild asthmatics there is no depletion of peripheral or sputum basophils post-allergen provocation (Gauvreau et al., 2021). Although benralizumab uses an alternative approach of targeting the IL-5Rα, there still remains an incomplete ablation of exacerbation rates, similar to mepolizumab and reslizumab (**Table 4**).

3.2.1 Possible Reasons for Suboptimal Responses With Anti-IL-5 Pathway Biologics

This brings to question why there is only a partial reduction in exacerbation rates in severe asthmatics treated with anti-IL-5RAα biologics. There are several reasons for sub-optimal or failure of response which will be described below. First, it is important to understand that severe asthmatics eligible for biologics are already on ICS therapy. We know from multiple reports that nonadherence to ICS in severe asthmatics is substantial (Gamble et al., 2009; Lee et al., 2018; Sulaiman et al., 2018), and that nonadherent patients receiving mepolizumab have worse clinical outcomes (d'Ancona et al., 2020). This brings up to two interesting propositions. The first is that worsened underlying disease as a result of noncompliance with ICS is associated with uncontrolled inflammation, and this may make it more difficult for biologics targeting eosinophils to have a noticeable clinical impact in patients with suboptimal responses. Secondly, it is important to emphasize that corticosteroids target other aspects of T2-high inflammation that are not inhibited by anti-IL-5 biologics, including IL-4 and IL-13 activity (Ray et al., 2016). One can postulate that if additional T2-high inflammatory pathways beyond the IL-5 pathway are kept under control in

TABLE 6 | Summary of randomized clinical trials assessing tezepelumab in severe asthma.

Anti-TSLP: tezepelumab

Landmark study and year	RCT phase	Asthma severity	Biomarker	Dosing, duration and route of administration	Clinical effect	Molecular effect
Gauvreau et al. (2014)	Phase 1b	Mild allergic asthma	Atopic, independent of PB Eosinophils	Dosing: 700 mg Q4W Route: SQ Duration: 12 W	34% improvement in FEV_1 on day 84 ($p = 0.02$) compared to placebo	- PB eosinophils declined post-dosing and reached normal levels by 4 W - SP eosinophils reached normal levels by 6 weeks - FeNO levels improved 1 W post-first dose - No effect on total IgE levels
PATHWAY, 2017, 2021, 2021; Corren et al. (2017); Corren et al. (2021a); Corren et al. (2021b)	Phase 2b	Severe uncontrolled asthma	PB Eosinophils ≥250 or <250 cells/μl	Dosing 700 mg Q4W 210 mg Q4W 280 mg Q2W Route: SQ Duration: 52 W	- 62–71% reduction in exacerbation irrespective of phenotype, across all seasons - Reduced asthma-exacerbation related hospitalizations - FEV_1 120–150 ml improvement vs. placebo (p = 0.002–0.015) -Significant improvement in ACQ and AQLQ scores in higher-dose intervention arms	- Decrease in PB eosinophils in all tezepelumab groups at 4 W onwards - Decrease in total serum IgE all tezepelumab groups
NAVIGATOR, 2021; Menzies-Gow et al. (2021)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells μl PB eosinophils	Dosing 210 mg Q4W Route: SQ Duration: 52 W	- Reduced AAER by 44–59% vs. placebo, irrespective of phenotype - FEV_1 230 ml improvement vs. placebo ($p < 0.0001$) - Significant improvement in ACQ and AQLQ	- Decrease in PB eosinophils and FeNO levels at 2 W onwards vs. placebo - Serum IgE levels reduced over 5 W vs. placebo
UPSTREAM (2021); Sverrild et al. (2021)	Phase 2	Severe uncontrolled asthma	Independent of blood eosinophils or atopic status	Dosing: 700 mg Q4W Route: SQ Duration: 12 W	- Mean change in PD_{15} significantly reduced - Non-significant improvement in ACQ	- Airway tissue and BAL eosinophils decreased by 74 and 75%, respectively (p = 0.004, p = 0.01) - No significant change in tissue mast cells - Subepithelial neutrophils increased by 51% with tezepelumab vs. 33% in placebo (non-significant)
CASCADE, 2020 (Ongoing), 2021 Diver et al. (2021)	Phase 2	Moderate-severe uncontrolled asthma	<300 cells/μl or ≥300 cells μl PB eosinophils	Dosing: 210 mg Q4W Route: SQ Duration: 28 W	-Reduced AHR to mannitol vs. placebo	- Decreased submucosal eosinophils vs. placebo, regardless of baseline PB eosinophils - No difference in $CD3^+$ T cells or $CD4^+$ T cells, mast cells - No difference in reticular basement membrane thickness and epithelial integrity
SOURCE, 2020 (Ongoing) Wechsler et al. (2020b)	Phase 3	Severe uncontrolled asthma	<300 cells/μl or ≥300 cells μl PB eosinophils	Dosing: 210 mg Q4W Route: SQ Duration: 48 W	Pending	Pending
DESTINATION, 2020 (Ongoing) Menzies-Gow et al. (2020)	Phase 3	Severe Uncontrolled Asthma	<300 cells/μl or ≥300 cells μl PB eosinophils	Dosing: 210 mg Q4W Route: SQ Duration: 36 W	Pending	Pending

TABLE 7 | Summary of IL-33/ST2-targeted therapy in severe asthma.

Anti-IL-33/ST2						
Landmark study and year	**RCT phase**	**Disease model**	**Disease phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
Chen et al. (2019)	Phase 2a	Atopic dermatitis	Atopic	Biologic: Etokimab Dosing: 300 mg Route: IV	-83% achieved EASI50 and 33% EASI75	- Reduction in PB eosinophils at day 29 - Reduction in skin neutrophil infiltration post-HDM skin challenge - Inhibited neutrophil migration to skin
Chinthrajah et al. (2019)	Phase 2a	Peanut allergy	Atopic	Biologic: Etokimab Dosing: 300 mg Route: IV Duration: 6 W	- Significant desensitization to peanuts	- Reduction in cytokine levels (IL-4, IL-5, IL-9, IL-13), and ST2 levels in $CD4^+$ T cells in PB - Reduction in IgE at day 15
Wechsler et al. (2021)	Phase	Moderate-severe asthma	<200 cells/μl or ≥200 cells μl PB eosinophils	Biologic: Itepekimab Dosing: 300 mg Route: SQ Duration: 12 W	- Reduction in loss of asthma control (22%) - No improvement in FEV1 - Improvement in AQLQ and ACQ	- Reduction in mean blood eosinophil count, FeNO, serum total IgE, periostin, plasma eotaxin-3, and serum pulmonary and activation-regulated chemokine (PARC)
NCT03207243, 2020 (Ongoing)	Phase 2a	Moderate-severe asthma	Independent of blood eosinophils or atopic status	Biologic: GSK3772847 Dosing: 10 mg/kg Q4W Route: IV Duration: 28 W	Pending	Pending
NCT02918019, 2020 (Ongoing)	Phase 2b	Severe uncontrolled asthma	Independent of blood eosinophils or atopic status	Biologic: MSTT1041A Dosing: 210 mg Q4W Route: SQ Duration: 50 W	Pending	Pending

ICS-treated patients, then the beneficial effects of anti-IL-5 biologics may be amplified. Thus, continued and proper use of ICS is critical to dampening multiple T2-high endotypes that are not specifically targeted with anti-IL-5 biologics.

Now for a second proposal, there may be other inflammatory cells and cytokines, outside of the eosinophil-IL-5 pathway, that are equally, if not more important. For example, ILC2s, potent sources of IL-5, IL-9, IL-13, and PGD2, have been found to be higher in blood and sputum of severe asthmatics on high-dose steroids compared to mild asthmatics (Smith et al., 2016), and that these numbers are even higher in those with uncontrolled eosinophilia despite OCS. With respect to specifically targeting ILC2s, there have been a number of studies which have assessed the effect of corticosteroids and biologics on ILC2 numbers in severe asthma. Treatment with ICS has been shown to reduce ILC2-mediated inflammation, as well as ILC2 in nasal polyps, peripheral blood and sputum in asthma and asthma with allergic rhinitis (Walford et al., 2014; Yu et al., 2018). The corticosteroid-responsiveness of ILC2s may be dependent on activation by upstream cytokines including IL-33 and TSLP. This is supported by *in vitro* findings that IL-5 production from IL-33-induced ILC2s can be attenuated by corticosteroids, but not when ILC2s are stimulated by TSLP (Liu et al., 2018). Not unexpectedly, although anti-IL-5 agents reduce total sputum IL-5 levels, they do not attenuate ILC2s within sputum or blood, suggesting that these biologics neutralize IL-5 production from these cells but do not affect their overall function within the airways (Sehmi et al., 2018; Mukherjee et al., 2018b). Given the importance of ILC2s in asthma, there is a need to develop treatments that specifically target the function of these cells, and as *in vitro* studies have shown, this may be done most effectively at an upstream level with targeting of alarmins. Another consideration is that there are alternative signalling pathways, independent of alarmins, that can activate ILC2s, including the TNF superfamily pathway, including the TL1A/DR3 axis (Machida et al., 2020). Sputum TL1A levels are present in approximately 50% of prednisone-dependent severe asthmatics with uncontrolled eosinophilia (Machida et al., 2020). TL1A-induced activation of ILC2s, in the presence of TSLP and IL-2 is not responsive to dexamethasone (Machida

TABLE 8 | Summary of other therapeutic targets in severe asthma.

Other agents						
Landmark study and year	**RCT phase**	**Disease severity**	**Disease phenotype**	**Dosing, duration and route of administration**	**Clinical effect**	**Molecular effect**
KRONOS, 2020 Levine et al. (2020) Anti-Siglec 8	Phase 1b	Severe allergic conjunctivitis	Atopic	Biologic: AK002 Dose: 0.3, 1, 3 mg/kg q4W Route: IV Duration: 6 M	- ACQ score improved by 74% vs. placebo - 72% reduction in asthma sx	-N/A
Hirano et al. (2020) Anti-Siglec 8	Phase 2	Eosinophilic gastritis and esophagitis	Atopic	Biologic: AK002 Dose: 0.3, 1, 3 mg/kg q4W Route: IV Duration: 4 M	- Improvement in dysphagia symptom scores	- Reduction in esophageal eosinophils
EXHALE, 2017 Prussin et al. (2017) Dexpramipexole	Phase 2	Moderate-severe asthma	≥300 cells µl PB eosinophils	Dose: 75–300 mg/day Route: PO Duration: 12 W	- Improved Pre-BD FEV_1 from baseline	- Reduced PB eosinophils at 12 W
LUSTER 1&2, 2021 Brightling et al. (2021b) Fevipiprant	Phase 3	Severe Asthma	≥250 or <250 cells µl PB eosinophils	Dose: 150–450 mg/day Route: PO Duration: 52 W	- Improved AAER in eosinophil high patients	- N/A
Gonem et al. (2016) Fevipiprant	Phase 2	Moderate-Severe Asthma	≥2% SP eosinophils	Dose: 250 mg BID Route: PO Duration: 12 W	- Favourable safety profile	- 4.5 times reduction in SP eosinophils
Bateman et al. (2017) Fevipiprant	Phase 2	Moderate-Severe Asthma	IgE ≥0.35 IU mEq	Dose: 1–450 mg OD or BID Route: PO Duration: 12 W	- Improved pre-BD FEV_1 at 12 W	- N/A
Brightling et al. (2021a) Risankizumab	Phase 2	Severe asthma		Dose: 90 mg q4W Route: SC Duration: 24 W	- Shorter time to asthma worsening	- No change in blood eosinophils, sputum eosinophils or neutrophils

et al., 2020). This further substantiates the heterogeneity of asthmatics, and highlights the hypothesis that the cytokine milieu within the airway determines responsiveness to corticosteroids and biologics. Aside from ILC2s, there are other important immune pathways to consider. For example, basophils have been shown to produce marked levels of IL-4 and IL-13 within the airways (Salter et al., 2015; Salter et al., 2016). Basophils are not only activated through an IgE-dependent pathway but also by alarmin cytokines including TSLP, IL-33, and IL-25 (Salter et al., 2015; Salter et al., 2016). Interestingly, in severe asthma, there is increased expression of receptors for IL-33 and IL-25 on basophils, particularly after IgE stimulation (Boita et al., 2018). Thus, alarmin cytokines can not only activate basophils in an IgE-independent manner, but IgE itself can upregulate receptor expression for alarmin cytokines, creating a vicious cycle. Although basophils have been found to express the IL-5 receptor, there is mixed evidence as to whether anti-IL-5 agents can affect basophil function. For example, Wright et al. (2019) found that 16 weeks of treatment with mepolizumab does not affect blood basophils in severe asthma. Similar to basophils, mast cells (MC) undergo extensive degranulation in fatal asthma suggesting that these cells are highly activated in severe asthma. Through both IgE-dependent and -independent mechanisms, MC not only release mediators such as histamine, prostaglandin and leukotrienes, but also produce a wide range of cytokines, including IL-4, IL-13, IL-6, IL-17, and TSLP (Bradding et al., 1992; Bradding et al., 1994; Ying et al., 1995; Ying et al., 2005). Collectively, there are numerous immune cells, outside of eosinophils, which can contribute to inflammatory processes inherent in severe asthma, independent of IL-5. Lastly, in addition to cytokines, there are granule proteins produced by eosinophils that play an important role in promoting airway obstruction. For example, EPX, a product of eosinophils, utilizes respiratory burst-derived H_2O_2 to generate reactive oxidants that can kill pathogens or activate airway cells (Wu et al., 2000). EPX has been shown to promote mucus plug formation by generating

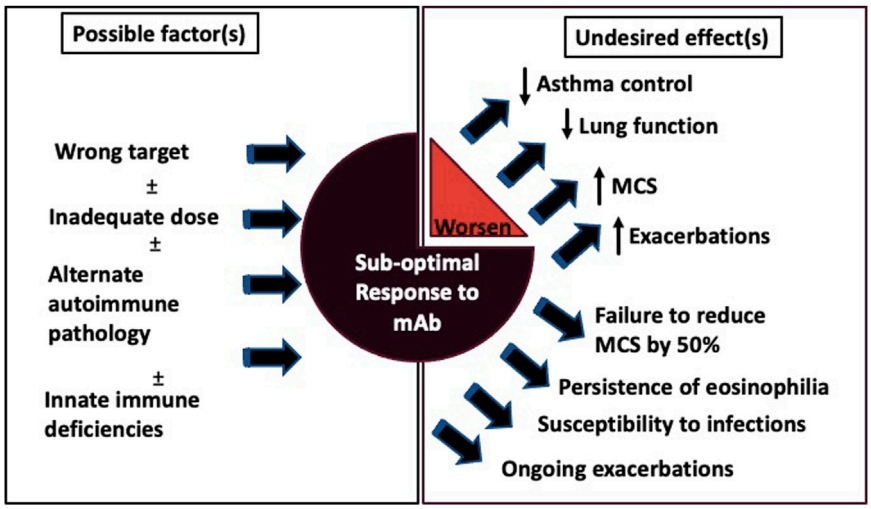

FIGURE 2 | Factors affecting optimal biologic response and clinical ramifications. The schematic addresses the primary factors that alone or in combination can lead to sub-optimal treatment response to currently approved monoclonal antibodies (mAbs) in severe asthma. The sub-optimal responses to mAbs can result in undesired clinical manifestations *via*, persistence and worsening of asthma symptoms, exacerbations, infections and decline in lung functions. Abbreviations: mAb: Monoclonal anitbody; MCS: Maintenance corticosteroids.

oxidants that modify mucins. Mucus plugging has been found in severe asthmatics and may predispose to infection (Dunican et al., 2018). Biologic therapy may not effectively target mucus plugging and is important to identify in patients with recurrent infections.

Other reasons for underlying suboptimal responses to biologics are related to dosing regimen and routes of administration. Mepolizumab has fixed dosing with SC administration versus reslizumab with IV weight-based dosing. Notably, the clinical benefits are the same for high and low doses of mepolizumab that is administered either IV or SC in severe asthmatics requiring high dose ICS (Mukherjee et al., 2018b). Conversely, severe asthmatics requiring daily prednisone have better clinical outcomes with higher doses of IV over lower doses of SC administration (Mukherjee et al., 2018b). These findings may be explained by lower doses administered SC not adequately neutralizing IL-5 within the airways, despite attenuated peripheral eosinophilia. For example, patients treated with 4 doses of weight-adjusted IV reslizumab after previously being treated with SC mepolizumab, resulted in suppressing both airway and peripheral eosinophilia (Mukherjee et al., 2018b). The magnitude of attenuation with reslizumab was greater compared to treatment with 12 doses of mepolizumab. The suppression of eosinophils and progenitors coincided with clinical improvement, as shown by increased FEV_1 and asthma control. Patients who did not respond to anti-IL-5 treatment had higher sputum IL-5 levels. Collectively, this suggests that a greater concentration of anti-IL-5 either through increased dose or IV administration, is needed to neutralize T2-high inflammation, that is driven not only by eosinophils but also ILC2s, in peripheral and local airway compartments. Another hypothesis to consider is that low doses of anti-IL-5 biologics may cause worsening of airway eosinophilia through inducing immune-complex (IC) formation or complement consumption (Mukherjee and Nair, 2018; Mukherjee et al., 2020). ICs may act like cytokine depots, leading to increased potency of bound IL-5, resulting in worsening symptoms. We have seen detectable levels of sputum immunoglobulin-bound IL-5 in mepolizumab non-responders, coinciding with increased sputum IL-5. We only observed this immunoglobulin-IL-5 phenomenon in one patient receiving reslizumab, suggesting that this issue is prevented with higher dosing and/or IV delivery.

This leads us to the next hypothesis that inadequate dosing of IgG1 neutralising antibodies have the potential for disease worsening, particularly in those with an underlying airway autoimmune component. There is emerging evidence that local airway autoimmune responses contribute to corticosteroid insensitivity in severe asthma (Mukherjee et al., 2017; Mukherjee and Nair, 2018). A third of eosinophilic asthmatics have airway autoimmune responses, manifesting as auto-IgG antibodies directed against cell-derived granule proteins including, EPX (Liu et al., 2018). Severe asthmatics with sputum autoantibodies have characteristic clusters of sputum FEGs, indicative of active eosinophil cytolysis (Liu et al., 2018). Interestingly, we have shown that patients treated with reslizumab had a reduction in sputum anti-EPX IgG and antinuclear antibody (ANA), but not with mepolizumab. In fact, an increase in sputum anti-EPX IgG was seen in 66% of non-responders to mepolizumab. An additional study supports these findings, where 60% of severe asthmatics treated with mepolizumab showed a suboptimal response versus 32% with reslizumab (Mukherjee et al., 2020). In those who showed a poor response to mepolizumab, 23% worsened clinically, and had higher levels of sputum Anti-EPX IgG levels. An extension of this study showed that 43% of patients on either mepolizumab or reslizumab had suboptimal responses, with 14% of these patients having worsening asthma (Mukherjee et al., 2020). We found increased sputum IL-5, anti-EPX, EPX, and C3c in those with suboptimal response to biologics. In addition, increased C1-q/IgG levels and C1-q-IgG/IL-5-IgG dual-positive cells in sputum plugs were found in those who worsened on mepolizumab. This is supported by a case report

where we described a severe asthmatic treated with mepolizumab had worsening of symptoms, and molecular analysis revealed increased anti-EPX and IL-5^{+}ILC2s, suggesting that increased Th2 signaling leads to activation of IL-5-producing ILC2s and subsequent eosinophilia (Mukherjee et al., 2017). This brings up an interesting theory that inadequate drug dosing results in hetero immune-complex formation of complement-fixing antibody that is bound to the C1q molecule (Duncan and Winter, 1988), which can induce the complement cascade and promote recruitment of immune cells *via* the FcγR receptor (Stokol et al., 2004). As such, there may be an autoimmune-triggered IC-mediated phenomena in those with worsened response to mepolizumab. Finally, the monitoring of blood eosinophils did not help to identify this subgroup, nor did assessment of autoimmunity biomarkers. Peripheral eosinophilia was only observed in 8% of suboptimal responders, whereas 6% continued to have increased sputum eosinophils >3%, representing non-attenuated airway eosinophilia, and 69% of these patients had sputum eosinophils despite normal blood eosinophils. These findings support a discordance between blood and sputum eosinophils, and while peripheral eosinophils may be adequate for selecting patients that may benefit from anti-IL-5 therapy, it may be insufficient for monitoring therapeutic response to anti-IL-5 biologics (Mukherjee and Nair, 2015; Mukherjee et al., 2017; Mukherjee et al., 2018b; Drick et al., 2018; Ojanguren et al., 2018). Instead, we propose that sampling from the airways may be a more adequate way to identify monitor treatment response. For example, we have shown that sputum eosinophil count prior to treatment did not predict response to mepolizumab, nor was this the case with peripheral eosinophils. However, sputum eosinophils were effective for assessing response to treatment as early as 4 months post-treatment (Mukherjee et al., 2020). Of note, there is the emerging concept of "breathomics," which is the phenotyping of patients through non-invasive identification of exhaled volatile organic compounds (VOCs) using gas chromatography and mass spectrometry (De Vries et al., 2018; Sterk, 2019). Measurement of VOCs has been shown to have similar accuracy to sputum cell counts and FeNO (Sterk, 2019). This may be an alternative or additive approach to monitoring treatment response to biologic agents.

There is a subset of severe asthmatics with frequent respiratory infections that is thought to be secondary to underlying airway neutrophilia. There are higher levels of IgM and IgG in asthmatics compared to healthy controls with recurrent respiratory tract infections (Ho et al., 2020). Specifically, eosinophilic asthmatics have lower levels of IgA compared to healthy controls. We have shown that administration of IVIg leads to increased total IgG and subtypes, and these patients had fewer infective exacerbations over 12 months (Ho et al., 2020). These findings suggest that although eosinophilic inflammation may be dampened by biologics, exacerbations continue to occur due to underlying neutrophilia and humoral deficiency. It is important to consider assessing immunoglobulin levels in those with frequent asthmatics and replace if necessary. We have previously assessed severe asthmatic responses over 14 months to benralizumab, and found that 27% of patients had suboptimal responses and 40% of these patients had worsening disease (Poznanski et al., 2021). Only two patients with worsening asthma had sputum eosinophilia, whereas 16 had evidence of infective exacerbation with neutrophilic inflammation. A suboptimal response to benralizumab has been proposed to be due to impaired NK function and/or number (Poznanski et al., 2021). Overall, respiratory infections increased with benralizumab and had associated sputum neutrophilia, which is in contrast to mepolizumab or reslizumab that is associated with eosinophilic exacerbations. Previous history of infections predicted poor responses to benralizumab (Poznanski et al., 2021). Lastly, benralizumab is seemingly more potent than the other anti-IL-5 agents at suppressing airway eosinophilia. Although there have been no head-to-head trials with anti-IL-5 agents, benralizumab appears to be non-superior relative to mepolizumab or reslizumab from a clinical standpoint. As mentioned earlier, there are numerous other pathways, aside from the IL-5-eosinophil pathway which may contribute to asthma pathogenesis but may not be adequately attenuated by IL-5-targeted biologics. For example, alternative Th2 cytokines, such as IL-4 and IL-13 or alarmin cytokines, and other immune cells such as basophils, Th2 cells, MC, and ILC2s may still be present and activated within the airways despite treatment with benralizumab. Thus, regardless of complete attenuation of eosinophils, there are other, redundant pathways that can carry out airway inflammation. The combination of anti-IL-5 agents with biologics that target other important Th2 pathways may confer better clinical outcomes, however this needs to be studied in more detail in the future.

3.3 IL-4/IL-13 Targeted Therapy

In order to understand the efficacy of IL-4/IL-13 agents, it is important to understand receptor signaling involved with these two cytokines. IL-13 signals through the IL-13 receptor, of which there are two subtypes, including IL-13Rα1 and IL-13Rα2. IL-13Rα1 binds to IL-13 with low affinity, but when the IL-4 receptor, IL-4Rα1, joins to form a heterodimer, IL-13 is bound with greater affinity. The IL-13Rα2, binds to IL-13 with high affinity but lacks a cytoplasmic domain thus does not signal downstream, however it may act as a negative regulator of IL-13 and IL-4 signaling. While isolated blockade of either IL-4 or IL-13 has not been shown to be effective in treatment of severe asthma, dual blockade of IL-4 and IL-13 has shown promise.

With respect to anti-IL-13 biologics, two agents have been studied, lebrikizumab and tralokinumab. Studies looking at moderate-severe asthma with T2-high inflammation (total IgE ≥100 IU/ml and blood eosinophils ≥140 cells/μl) have shown that treatment with lebrikizumab resulted in 60% reduction of AAER and improved FEV_1, but no effect on symptoms (Corren et al., 2011). Subgroup analysis showed that patients with higher serum periostin (≥50 ng/ml) or FeNO had greater improvement in lung function. Furthermore, there was an observed decrease in FeNO and serum IgE, but not eosinophils. Larger RCTs have shown that severe asthmatics with T2-high biomarkers (serum periostin ≥50 ng/ml and/or blood eosinophils ≥300 cells/μl) treated with lebrikizumab had reduced AAER but no coinciding improvement in symptom scores and only marginal improvement in FEV_1 (Hanania et al., 2015; Hanania et al., 2016). Similarly, the majority of studies with tralokinumab did not show promising clinical outcomes (Piper et al., 2013; Brightling et al., 2015; Panettieri et al., 2018; Russell et al., 2018). Collectively, anti-IL-13-specific agents are not effective in treating severe asthma. This may be due to IL-13 primarily being involved with AHR as opposed to exacerbation and/or inflammation.

With respect to tralokinumab, it targets both the IL-13Rα1 and IL-13Rα2 subunits, and thus may dampen the anti-inflammatory effect through IL-13Rα2 (**Table 5**).

The trials with respect to anti-IL-4 biologics have also been disappointing. Pascolizumab was shown to be well tolerated in animal studies with monkeys and effective in neutralizing bioactivity of IL-4 (Hart et al., 2002). Human RCTs have shown that treatment with Altrakincept, a nebulized anti-IL-4 agent, in moderate-severe asthmatics significantly improved in FEV_1 and reduced FeNO, with no effect on AAER (Borish et al., 1999; Borish et al., 2001). Overall, anti-IL-4 agents have not yielded sufficient clinical efficacy to warrant further investigation and research has been halted. The lack of efficacy, may in part, be due to redundancy provided by IL-13, which signals through the same heterodimer.

Given the lack of impressive data from anti-IL-4 and IL-13 individual biologic agents, it was thought that perhaps targeting a common pathway between both cytokines may yield greater effect. Dupilumab is the first dual IL-4/IL-13 biologic approved for asthma treatment. It targets the shared IL-4Rα receptor and thus blocks signalling of both IL-4 and IL-13. An initial trial with dupilumab treatment in eosinophilic asthmatics (blood eosinophils ≥300 cells/μl or sputum eosinophils ≥3%) resulted in an 87% reduction in AAER (Wenzel et al., 2013). Of note, the treatment groups were instructed to stop their maintenance LABA at week 4 and wean from ICS from weeks 6–9. Exacerbations were only seen after the point of inhaler withdrawal, suggesting that dupilumab may be acting on the same pathway as ICS/LABA inhalers and hence has a redundant effect. A larger RCT with severe asthmatics (blood eosinophils ≥300 cells/μl) over a 24-week period showed 81% reduction in AAER compared to 60% reduction with the low eosinophilia group, both of which were significant compared to placebo. These findings suggest that dupilumab may be effective regardless of eosinophilic status. However, other RCTs showed that dupilumab treatment only yielded significant reduction in AAER in patients with blood eosinophils ≥150 cells/μl and FeNO ≥25 ppb. In terms of biomarkers to monitor response, dupilumab induces transient increases in blood eosinophils but significant reduction in FeNO, suggesting that FeNO may be a better biomarker to assess treatment eligibility and efficacy for this treatment.

3.3.1 Possible Reasons for Suboptimal Responses with Anti-IL-4/IL-13

There have been some case reports of adverse events when switching from anti-IL-5 to anti-IL-4/IL-13. For example, patients switched from anti-IL-5 to anti-IL-4/IL-13 had worsening in asthma control and showed increased use of OCS, with substantial increases in peripheral eosinophils (Eger et al., 2021). The reason for this worsening is not entirely understood, but the working hypothesis is that these patients had underlying anti-neutrophil cytoplasmic antibody (ANCA) negative EGPA triggered by the rebound hypereosinophilia brought on by dupilumab. We propose that both pathways (IL-5, IL-4/IL-13) will need to be targeted to allow for optimal disease control. Unfortunately, no studies thus far have looked at the efficacy of combining anti-IL-4/IL-13 and anti-IL-5 biologics for asthma treatment.

Sub-optimal responses to anti-IL-4/IL-13 may be explained by these agents primarily focusing on reducing AHR as opposed to dampening airway inflammation (Gour and Wills-Karp, 2015). We propose that dupilumab should be used in patients who have symptoms of AHR, and if there is overlap with airway inflammation, it may be reasonable to pair with an upstream inhibitor, such as an anti-IL-33 or anti-TSLP agent. In addition, it is well known that mucus hyperplasia is promoted by the IL-4/IL-13 axis (Munitz et al., 2008; Bao and Reinhardt, 2015) and as such, patients with mucus hypersecretion as a primary symptom should be managed with agents targeting this axis such as dupilumab.

Interestingly, Wechsler et al. assessed the treatment of severe asthmatics with a combination of dupilumab and anti-IL-33 (Wechsler et al., 2020a). The anti-IL-33 biologic on its own was able to improve asthma control and lung function, but this was not synergistic when combined with dupilumab. This may have been due to both agents having redundant T2-high pathways, whereas there was insufficient targeting of IL-5 or TSLP, and hence continued activity of ILC2s and Th2 cells. Alternatively, this study was not adequately powered for between group comparisons. This study certainly provides food for thought and warrants further investigation with respect to studying combined biologic regimens.

3.4 Alarmin Cytokine Therapy

The development of anti-alarmin biologics has been one of the most exciting innovations in asthma therapy to date. Tezepelumab is a human IgG_2 antibody directed against TSLP, that can be administered IV or SC. The first landmark trial to assess the efficacy of tezepelumab was carried out in mild asthmatics, which showed significant improvement in FEV_1 and reduction of peripheral and sputum eosinophils, along with decreased FeNO levels during the late phase response post-allergen provocation (Gauvreau et al., 2014). Subsequent RCTs in severe asthmatics showed that tezepelumab treatment resulted in a 44–71% reduction in AAER, irrespective of baseline peripheral eosinophilia (**Table 6**) (Marone et al., 2019; Corren et al., 2021a; Menzies-Gow et al., 2021; Sverrild et al., 2021). Patients were also shown to have improvements in FEV_1 and symptoms, with a coinciding reduction in peripheral eosinophils and serum IgE. Collectively, these data suggest that targeting upstream cytokines, such as TSLP, may prove to be beneficial in multiple asthma endotypes, both within and outside of T2-high inflammation.

Initial studies that looked at anti-IL-33 agents were done in other sites of allergy beside asthma, specifically in atopic dermatitis and peanut allergy. Etokimab has been shown to improve symptoms related to atopic dermatitis and reduce desensitization to peanuts (Chen et al., 2019; Chinthrajah et al., 2019). These clinical findings were associated with significant reductions in peripheral eosinophils and T2 cytokine levels, along with total serum IgE. There are multiple RCTs underway assessing the efficacy of anti-IL-33 biologics to treat asthma, but most results are pending (**Table 7**). Of note, the first RCT was recently published in 2021 by Wechsler et al. which assessed the effect of an anti-IL-33 agent called itepekimab in a phase 2 trial with moderate-to-severe asthma (Wechsler et al., 2021). They found that following 12 weeks of treatment with itepekimab there was an improvement in asthma control and quality of life. Furthermore,

loss of asthma control occurred in 22% of patients in the itepekimab group compared to 19% in the dupilumab group and 27% in the combined group. Interestingly, itepekimab alone, or in combination with dupilumab resulted in decreased blood eosinophil. Itepekimab alone was also able to reduce FeNO, serum total IgE, periostin, and plasma eotaxin-4 but to a lesser extent compared to dupilumab or combined therapy. Overall, this anti-IL-33 agent reduced blood eosinophils to a lesser extent than anti-IL-5 agents and it had reduced effect on eotaxin-3, which is an IL-13 product, compared to dupilumab. These findings suggest incomplete inhibition of Th2 inflammation with blockade of IL-33, given that other alarmin cytokines are still active, including TSLP. More studies are needed to determine the effect of anti-IL-33 agents on airway eosinophils and other inflammatory parameters.

Overall, there have been not been sufficient RCTs with anti-alarmin agents to be able to identify suboptimal or failure to respond and what might be responsible for these outcomes. It will be helpful in the future to assess dual targeting of upstream and downstream inflammatory cytokines to treat severe asthma.

3.5 Other Current Therapy

There have been other agents that target various aspects of eosinophil function which have been assessed, albeit less extensively than the aforementioned biologics (**Table 8**). Sialic-acid-binding immunoglobulin-like lectin (Siglec)−8 is a cell surface receptor found on mast cells and eosinophils. Initial studies have shown that an anti-Siglec-8 induces death of cytokine-primed eosinophils and inhibits IgE-mediated mast cell activation (Levine et al., 2020). RCTs with AK002 in severe allergic conjunctivitis reported improved symptoms (Levine et al., 2020), and these patients were also found to have comorbid asthma, with 72% reduction in asthma symptom scores. Hirano et al. assessed AK002 in eosinophilic gastritis and esophagitis, showing significant improvement in dysphagia symptoms and reduction in esophagus eosinophils (**Table 8**) (Hirano et al., 2020). Further studies are needed to determine the efficacy of anti-Siglec-8 in asthma.

Dexpramipexole is a small molecule traditionally developed as treatment for amyotrophic lateral sclerosis. These studies showed significant and persistent reduction in blood eosinophils 1–2 months after drug initiation. The spotlight has now turned to assessing the use of dexpramipexole for asthma and CRS. Initial studies with CRS have shown that dexpramipexole can reduce peripheral eosinophils to $<0.020 \times 10^9$/L at month 6 post-initiation, and yield a 97% reduction in nasal polyp tissue eosinophils from baseline (Laidlaw et al., 2019). Similarly, another study showed that dexpramipexole can reduce blood eosinophils by 93% compared to baseline at month 6 and 94% in tissue nasal polyps (Prussin et al., 2017). In terms of asthma, 12 weeks treatment with dexpramipexole resulted in significant reduction of peripheral eosinophils and improved FEV_1 (Prussin et al., 2021). More data is needed to determine effect on sputum eosinophils and whether this reduction translates into meaningful clinical outcomes.

Prostaglandin D2 receptor 2 (PGD2) is a potent mediator involved in asthma pathogenesis with the main function to promote airway smooth muscle contraction. In addition, PDG2 activates the DP2 chemokine receptor, also known as CRTH2, on Th2 cells, ILC2s, granulocytes, and monocytes. The PGD2/CRTH2 axis is implicated in cell adhesion, survival, and activation resulting in cytokine/chemokine production, and subsequent downstream eosinophilia. Fevipiprant is an oral PGD2 receptor antagonist recently developed for asthma. Studies have shown that in moderate-severe asthma fevipiprant reduces sputum and bronchial submucosal eosinophils, and reduces airway smooth muscle mass compared with placebo (Saunders et al., 2019). Similar studies have shown that fevipiprant can induce a 3–5 greater 3-5 fold reduction in sputum eosinophils (Gonem et al., 2016; Saunders et al., 2019). From a clinical perspective, treatment with fevipiprant can improve pre-dose trough FEV_1 and symptom scores, as well as reduce AAER (Erpenbeck et al., 2016). Conversely, other trials did not show improvement in symptom scores or FEV_1 (Castro et al., 2021). Overall, findings have been inconsistent with respect to clinical efficacy of fevipiprant treatment. A recent systematic review confirms this by showing that although the agent is safe, it does not reach minimal clinically important difference (Yang et al., 2021). Suboptimal responses may be explained by the PGD2/CRTH2 axis being one of the many pathways that control Th2 and ILC2 activation, and that anti-alarmin pathways may be more crucial to target. In summary, there is a need for more studies determining the efficacy of combining these agents with other biologics.

Lastly, IL-23 has been implicated in airway inflammation that is mediated by Th2 and Th17 cytokines. Animal models have shown IL-23 to promote Th17 cell proliferation, which in turn maintains IL-17A and IL-17F production and neutrophil recruitment (Li and Hua, 2014). IL-23 also promotes Th2 cytokine production and eosinophil infiltration (Wakashin et al., 2008). Brightling et al. (2021a) recently conducted a phase 2a RCT assessing the effect of an anti-IL-23p19 agent, called risankizumab in severe asthma. They found that the time-to-first asthma worsening and rate ratio for annualized asthma worsening was shorter in the risankizumab group compared to placebo. There was no effect of risankizumab on FeNO, median blood eosinophil count or sputum eosinophils and neutrophils. They did, however, report that risankizumab reduced the sputum IL-23 gene set and pathways associated with activation of cytotoxic T cells and NK cells. Their findings challenge the proposed role of Th17 and IL-23 in severe asthma. The worsening in asthma control may have been attributed to targeting of IL-23 leading to an increase in Th2 mediators, such as IL-13, thereby resulting in increased smooth-muscle tone within the airways. Interestingly, the poorer outcomes in the risankizumab group were amplified in those with higher blood eosinophils counts. Given the evidence of potential harm related to IL-23 blockade, it may be reasonable to steer away from this area of study or consider studies assessing the efficacy of combining anti-IL-23 or anti-IL-17 agents with Th2-targeted agents.

4 CONCLUSION

Overall, we have described a number of mechanisms that could be contributing to failed or sub-optimal response to biologic

therapy in severe asthma. The main themes are the need for proper dosing and route of administration of biologics, the identification of underlying inflammation through proper immune endotyping, as well as the targeting of multiple pathways, both upstream and downstream. An astute understanding of the molecular mechanisms and their associated clinical manifestations require careful consideration for development of valid biomarkers that will help guide optimal treatment and monitor therapeutic response. Moving forward, we need these biomarkers to assess individual patient symptoms and determine the underlying immunological mechanism that may be primarily responsible for driving disease severity and aid in choosing the right targeted therapy.

AUTHOR CONTRIBUTIONS

BS and MM wrote the manuscript draft. PL contributed to the final development of the manuscript. MM and PL conceived the review structure and take responsibility of the overall manuscript and information.

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