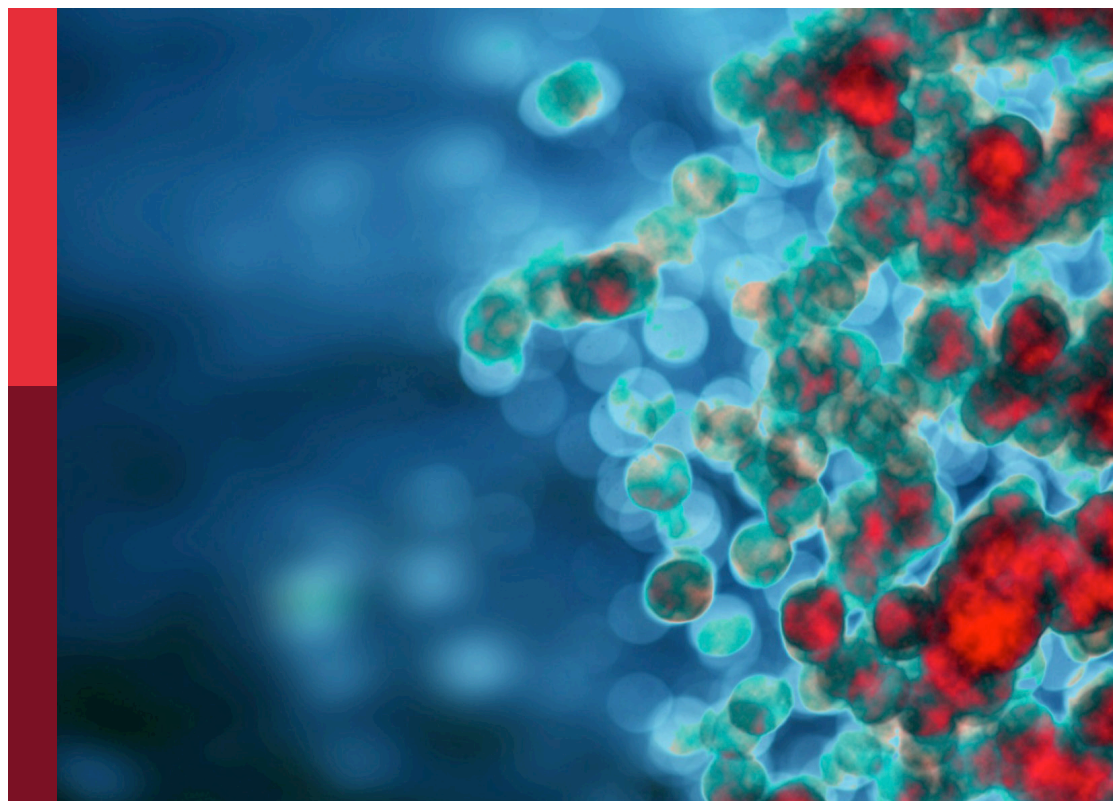# JAK inhibition in autoimmune and inflammatory diseases

Edited by
Jean-Baptiste Telliez, Massimo Gadina, Kamran Ghoreschi,
Olli Silvennoinen and Francesca Romana Spinelli

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JAK inhibition in autoimmune and inflammatory diseases

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Editorial: JAK inhibition in autoimmune and inflammatory diseases

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Editorial on the Research Topic
JAK inhibition in autoimmune and inflammatory diseases

The journey to characterize the central role of JAK/STAT signaling in regulating many aspects of normal or pathologic immune responses started about thirty years ago (1). Indeed, the discoveries of the JAK tyrosine kinases and STAT transcription factors (1) quickly unraveled that their function is required to transmit signal through type 1 and type 2 cytokine receptors (2). Since then, targeting intracellular signaling with Janus kinase (JAK) inhibitors has become a major and successful approach to treat many autoimmune and inflammatory diseases (3) with multiple JAK inhibitors approved or in late-stage clinical development for a wide array of such diseases. This short collection of articles further exemplifies the breadth and depth of the science being pursued to further advance the understanding of JAK/STAT signaling and the effects JAK inhibition in diseases as diverse as rheumatoid arthritis, inflammatory bowel disease, alopecia areata, multiple sclerosis and Sjögren's syndrome, Behçet's syndrome and vasculitis.

In this Frontiers in Immunology collection of articles, we have collected original works and reviews that provide new insights into the role of JAK/STAT signaling in various systems by using disruption of the signaling pathways with various pharmacological agents.

The work of Gopalakrishnan et al. explores the role of JAK/STAT signaling in intestinal epithelial cells (IECs) from colonoids, from UC patients or non-IBD controls, and how it regulates TNF+Poly(I:C)-dependent upregulation of MHC-II expression.

This is a good example illustrating how JAK/STAT signaling in non-immune cells could impact the adaptive immune response *via* antigen presentation.

The review by Watanabe and Hashimoto covers the pathophysiology of large vessel vasculitis, a severe inflammatory condition of the blood vessels. Starting from the studies done in patients with rheumatoid arthritis, and the fact that two diseases are driven by similar cytokines such as IL-6, IL-12 and type I and type II IFNs, the authors report about a preclinical model of vascular inflammation as well as few case reports in which JAK inhibitors have been demonstrated to decrease vascular inflammation. In particular, the data from the human studies, albeit with the caveat that have been collected in a limited number of patients, seems to suggest that JAK inhibitors could be employed to induce remission and to reduce the use of steroids.

Zhao et al reported a case series of 4 patient with Behcet's disease involving the gastrointestinal tract, successfully treated with tofacitinib. The intestinal involvement in Behcet's disease may be challenging and the treatment is not yet ideal, with some patients failing to achieve endoscopic remission with immunosuppressants and biological drugs (TNF inhibitors). All four patients had failed previous treatment with different immunosuppressive drugs and, after starting tofacitinib, showed clinical and histologic improvement that allowed them to reduce the dose of glucocorticoids.

The study reported by Hong et al. compared data collected from genome-wide association studies (GWAS) and from the Gene Expression Omnibus (GEO) database to assess susceptibility genes and differentially expressed genes (DEGs) in multiple sclerosis and Sjögren's syndrome. Interestingly, the authors found that among common susceptibility genes, several were associated with cytokines and the JAK/STAT signaling pathway. With this information in hand, the authors took advantage of the Comparative Toxicogenomics Database (CTD), DrugBank database, and Drug–Gene Interaction (DGI) Database to search for drugs that could be of potential use in these pathologies. Notably, over 130 drugs, including JAK inhibitors were found to be potentially useful in multiple sclerosis and Sjögren's syndrome.

Given their pleiotropic effects on immune and non-immune cells involved in the pathogenesis of rheumatoid synovitis and bone erosion, JAK inhibitors have been approved for Rheumatoid Arthritis for 10 years (4). Despite the remarkable effect of this class of drugs in many patients, still not all of them respond to the treatment. Looking for biomarkers of treatment response, Tucci et al. evaluated by flow cytometry the monocyte phosphorylation of STAT1, in basal conditions and after stimulation with IL-2, IFN-α, and IL-6, in a cohort of rheumatoid arthritis patients treated with baricitinib. The authors observed a significant reduction in monocyte number and cytokine induced STAT1 phosphorylation only in responder patients, suggesting that these changes could be an early predictor of treatment response.

Palmroth et al. studied the signaling effects of the JAK1/3 inhibitor tofacitinib in a cohort of patients with rheumatoid arthritis. They focused on the levels of constitutive and cytokine-induced phosphorylated STATs in monocytes, T cells and B cells isolated from peripheral blood. They found that tofacitinib improves rheumatoid arthritis and suppresses JAK-STAT signaling. The degree of the inhibition of STAT phosphorylation by the JAK inhibitor was dependent on the cytokine used for STAT activation and differences were also found in the indicated cell types studied. Based on their findings in a small sample size the authors speculate that the assessment of the baseline JAK-STAT signaling profile may have a prognostic value for the treatment responses to certain JAK inhibitors.

The review of Lensing and Jabbari tackles the pathogenesis of alopecia areata discussing the involvement of cytokine whose signaling relies on JAK/STAT and the rationale for targeting this pathway with first- and second-generation JAK inhibitors. In particular, the authors discussed the benefits and potential limits of JAK inhibitors in patients with alopecia areata.

Yu et al. report a case on the successful treatment of alopecia universalis in a patient who received the JAK1/3 inhibitor tofacitinib. The authors documented the clinical response and the levels of some cytokines in the patient's peripheral blood. Interestingly, they observed a strong increase in serum cytokine levels of most factors studied. So far, little data is available in the literature on serum cytokine levels during the treatment with JAK inhibitors like tofacitinib. In case there is accumulating data with similar observations in future, we need to understand why increasing cytokine levels in the blood are associated with a clinical improvement of an autoimmune disease like alopecia areata and if increased cytokine release is harmful for patients.

The basic research study by Dai et al. focused on the immunological effects of the JAK1/3 inhibitor ifidancitinib in a model of alopecia areata using C3H/HeJ mice. The authors studied the effects of ifidancitinib on cytokine-induced STAT activation in T cells and on T cell proliferation *in vitro* and *in vivo*. Ifidancitinib treatment reversed alopecia areata in C3H/HeJ mice. By elegant immunological analysis they describe that JAK1/3 inhibition regulates T cell exhaustion and suggest that this JAK/STAT-depending mechanism is responsible for the beneficial effects observed in mice with alopecia areata treated with ifidancitinib.

Finally, Iglesias et al. review recent developments on T cell costimulation blockade in treatment of transplant rejection. The focus is on mechanisms that limit CTLA-4-Ig efficacy, and it gives a thorough insight on potential complementary therapies, including JAK inhibitors to overcome the inflammation induced resistance.

Author contributions

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

Conflict of interest

J-BT is an employee of Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tofacitinib Suppresses Several JAK-STAT Pathways in Rheumatoid Arthritis *In Vivo* and Baseline Signaling Profile Associates With Treatment Response

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Objective: Current knowledge on the actions of tofacitinib on cytokine signaling pathways in rheumatoid arthritis (RA) is based on *in vitro* studies. Our study is the first to examine the effects of tofacitinib treatment on Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathways *in vivo* in patients with RA.

Methods: Sixteen patients with active RA, despite treatment with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), received tofacitinib 5 mg twice daily for three months. Levels of constitutive and cytokine-induced phosphorylated STATs in peripheral blood monocytes, T cells and B cells were measured by flow cytometry at baseline and three-month visits. mRNA expression of JAKs, STATs and suppressors of cytokine signaling (SOCS) were measured from peripheral blood mononuclear cells (PBMCs) by quantitative PCR. Association of baseline signaling profile with treatment response was also investigated.

Results: Tofacitinib, in csDMARDs background, decreased median disease activity score (DAS28) from 4.4 to 2.6 (p < 0.001). Tofacitinib treatment significantly decreased cytokine-induced phosphorylation of all JAK-STAT pathways studied. However, the magnitude of the inhibitory effect depended on the cytokine and cell type studied, varying from 10% to 73% inhibition following 3-month treatment with tofacitinib. In general, strongest inhibition by tofacitinib was observed with STAT phosphorylations induced by cytokines signaling through the common-γ-chain cytokine receptor in T cells, while lowest inhibition was demonstrated for IL-10 -induced STAT3 phosphorylation in monocytes. Constitutive STAT1, STAT3, STAT4 and STAT5 phosphorylation in monocytes and/or T cells was also downregulated by tofacitinib. Tofacitinib treatment downregulated the expression of several JAK-STAT pathway components in PBMCs,

SOCSs showing the strongest downregulation. Baseline STAT phosphorylation levels in T cells and monocytes and SOCS3 expression in PBMCs correlated with treatment response.

Conclusions: Tofacitinib suppresses multiple JAK-STAT pathways in cytokine and cell population specific manner in RA patients *in vivo*. Besides directly inhibiting JAK activation, tofacitinib downregulates the expression of JAK-STAT pathway components. This may modulate the effects of tofacitinib on JAK-STAT pathway activation *in vivo* and explain some of the differential findings between the current study and previous *in vitro* studies. Finally, baseline immunological markers associate with the treatment response to tofacitinib.

Keywords: rheumatoid arthritis, cytokines, JAK inhibitor, monocytes, T cells, B cells

INTRODUCTION

Cytokines are important mediators of inflammation and tissue destruction in rheumatoid arthritis (RA) (1, 2). Several cytokines involved in the pathogenesis of RA, such as interleukin-6 (IL-6), interferons (IFNs), granulocyte-macrophage colony-stimulating factor (GM-CSF) and common gamma chain cytokine family, act through Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (3, 4). JAK-STAT pathways consist of four JAK kinases [JAK1-3 and tyrosine kinase 2 (TYK2)] and seven signal transducers and activators of transcription (STAT1-6, including the homologs STAT5A and STAT5B). The signaling cascade is initiated by a cytokine binding to its receptor, which enables JAK activation by trans-phosphorylation. Subsequently, JAKs phosphorylate the receptor and STATs that dimerize, and translocate to the nucleus to regulate the expression of their target genes. Each cytokine receptor employs a specific combination of JAK kinases, e.g. IL-6 signals through JAK1 and JAK2/TYK2 and common gamma chain cytokines through JAK1 and JAK3 (5). JAK-STAT signaling pathway is under tight regulation, which involves e.g. proteins of suppressors of cytokine signaling (SOCS) family SOCS1-3 and cytokine-inducible SH2-containing protein (CIS1) (6).

We and others have demonstrated that certain JAK-STAT pathways are constitutively active in rheumatic diseases (7–10). In RA, STAT3 is constitutively phosphorylated in circulating T cells and monocytes, and this correlates with serum IL-6 levels, suggesting hyperactivation of the IL-6 –STAT3 axis (7, 9). In addition to STAT3, constitutive phosphorylation of STAT1 and STAT5 is increased in peripheral blood T cells from patients with active RA (7, 10). Constitutive STAT3 phosphorylation in circulating T cells of patients with recent-onset RA associates with disease activity and good treatment response to conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) (8). In addition, we have demonstrated that cytokine-induced STAT1 and STAT6 phosphorylation in circulating leukocytes associates with treatment response to biological drugs in established RA and to csDMARDs in recent-onset RA, respectively (11).

Tofacitinib, which inhibits JAK1, JAK3, and to a slightly lesser extent JAK2, was the first JAK inhibitor developed for treatment of RA (12, 13) and its efficacy is comparable to that of TNF-inhibitor adalimumab (14). Tofacitinib has been shown to inhibit

cytokine signaling and the effector functions of different immune cells and synovial fibroblasts *in vitro* (15–26) and in animal models of arthritis (15, 27, 28). However, information of the *in vivo* effects of tofacitinib on the activity of JAK-STAT pathways in RA patients is currently lacking and may differ from the results based on *in vitro* studies.

Gaining knowledge on how medicines, such as tofacitinib, function in patients with RA could help us to understand disease mechanisms, as well as both desired and undesired effects of JAK inhibitors. The current study is the first to investigate the JAK-STAT signaling profile by flow cytometry, and the expression of the signaling pathway components, in peripheral blood leukocytes of tofacitinib-treated RA patients. In addition, we examined associations between baseline immunological findings and the treatment response to tofacitinib.

MATERIALS AND METHODS

Patients

Patients fulfilling the 2010 ACR/EULAR classification criteria for RA were recruited for this clinical study in two rheumatology outpatient clinics (Tampere and Helsinki University Hospitals) between June 2018 and January 2020. Eligible patients had active disease at baseline visit: Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]) was >3.2 despite treatment with methotrexate and/or other csDMARDs. Key exclusion criteria were prior treatment with biologic therapies or JAK inhibitors, current infection, malignancy, severe hepatic impairment, pregnancy or lactation, hemoglobin <90 mg/dl, neutrophil count <1.0×10^9/l or lymphocyte count <0.75 x 10^9/l.

This study was approved by the National Committee on Medical Research Ethics (TUKIJA) and Finnish Medicines Agency Fimea and was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines. All patients gave their written informed consent.

Study Design

Graphical overview of the study is presented in **Figure 1A**. The study included a screening visit (0-3 months before baseline visit), a baseline visit (0 month) and a follow-up visit (3 months). Screening visit and baseline visit could be combined.

FIGURE 1 | **(A)** Graphical overview of the study and **(B–I)**. Flow cytometry gating strategy and examples of cytokine-induced phosphorylated STAT (pSTAT) in each cell population in one patient sample. **(B)** Monocyte gate was set based on CD33 positivity and light scattering characteristics (SSC-A). **(C)** Lymphocytes were gated based on light scattering characteristics (FSC-A and SSC-A). **(D)** Among lymphocytes, T cell gates were set to comprise $CD3^+CD4^+$ or $CD3^+CD4^-$ populations. **(E)** Among lymphocytes, B cell gate was set to comprise $CD3^-$ and $CD20^+$ population. **(F)** pSTAT1 histograms in unstimulated (open peak) and IFN-γ-stimulated (grey peak) monocytes. **(G)** pSTAT3 histograms in unstimulated (open peak) and IL-10-stimulated (grey peak) $CD4^+$ T cells. **(H)** pSTAT5 histograms in unstimulated (open peak) and IL-15-stimulated (grey peak) $CD4^-$ T cells. **(I)** pSTAT6 histograms in unstimulated (open peak) and IL-4 stimulated (grey peak) B cells. IFN, interferon; IL, interleukin, (p)STAT, (phosphorylated) signal transducer and activator of transcription.

Tofacitinib 5 mg twice daily was started at baseline visit and continued through the study. Patients continued their csDMARD therapy and prednisolone (0-10 mg/day) at a stable dose during the study.

The following patient-reported outcomes and clinical assessments were recorded at study visits: patient general health visual analogue scale (VAS) (0-100 mm), pain VAS (0-100 mm), health assessment questionnaire (HAQ) disability index (0-3), number of swollen and tender joints (46 joint count), physician's assessment VAS (0-100 mm) and DAS28. Tofacitinib treatment response was determined by the change from baseline DAS28-4[CRP].

Study-related blood samples were drawn at baseline and follow-up visits. Blood samples at the follow-up visit were taken 1-2 hours after the morning tofacitinib dose.

Cytokine-Stimulations and Flow Cytometry

Phosphorylation of STAT proteins, and levels of total STAT1 and STAT3 were studied using five-color flow cytometry. First, 100-μl aliquots of fresh blood samples were either left unstimulated or were stimulated by 100 ng/ml recombinant IL-2, IL-4, IL-6, IL-7, IL-10, IL-15, IL-21, IFN-α or IFN-γ (listed in detail in **Supplementary Table 1**) for 15 minutes at +37°C. The stimulation was terminated by transferring the samples on ice. Leukocytes were then immediately fixed and red blood cells lysed using BD Phosflow Lyse/Fix buffer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 10 min at +37°C, washed with PBS and permeabilized in ice-cold methanol for 10 min on ice followed by 1-4 weeks preservation in methanol at -80°C. In order to ensure the integrity of the results the samples that were collected in Helsinki were transferred in methanol on dry ice to Tampere, where sample preparation was continued and all stainings were performed. STAT phosphorylation levels were determined to be preserved for at least 4 weeks in methanol at -80°C in preliminary experiments (unpublished data).

Following two washes with FACS buffer (PBS supplemented with 0,1% bovine serum albumin and 0,01% sodium azide), samples were stained with fluorochrome-conjugated antibodies against CD33, CD3, CD4, CD20, STAT1, STAT3, phospho-STAT1 (pSTAT1), pSTAT3, pSTAT4, pSTAT5 and pSTAT6 (listed in detail in **Supplementary Table 2**) for 30 minutes at room temperature, protected from light. CD33 marker was selected to represent monocytes and CD20 to represent B cells, as the epitopes of more conventionally used markers CD14 and CD19, respectively, do not survive cold methanol permeabilization (29). Following antibody stainings the samples were washed twice with FACS buffer and fluorescence was measured with FACS Canto II (BD). To ensure consistent performance of the method throughout the study, 8-peak Rainbow calibration particles (BD) were used before every run.

Data acquisition was performed using FACSCanto II (BD) and the analysis of flow cytometer data using FlowJo Single cell analysis software (BD). CD4$^+$ and CD4$^-$ T cells were gated from the CD3$^+$ lymphocyte population, CD20$^+$ B cells from the CD3$^-$ lymphocyte population and CD33$^+$ cells represent monocytes (**Figures 1B–E**). A phycoerythrin (PE) fluorescence histogram was created for each cell population, and the median fluorescence intensity (MFI, arithmetic median) was calculated. Examples of cytokine-induced pSTATs are presented in **Figures 1F–I**. The change in STAT phosphorylation during the study was calculated by dividing the difference of fluorescence intensities at entry and after 3 months and dividing that by the fluorescence intensity at entry.

Peripheral Blood Mononuclear Cell Isolation, RNA Extraction, and qPCR Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077 medium

(Sigma Aldrich, St. Louis, MO, USA), washed twice with PBS containing 2 mM EDTA and snap frozen. Samples were collected at both locations but transferred from Helsinki to Tampere on dry ice for further preparations and analysis. Total RNA was extracted from PBMCs using the RNeasy Mini-Kit (Qiagen, Valencia, CA, USA). Total RNA (0.5 μg) was reverse-transcribed using M-MLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were performed by using HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Primer sequences for *STAT1, STAT3, STAT4, STAT5A, STAT5B, STAT6, JAK1, JAK2, JAK3, TYK2, SOCS1, SOCS2, SOCS3, CIS1* and *β-actin* are listed in **Supplementary Table 3**. The 10-μl real-time PCR reactions were performed with CFX384 (Bio-Rad Laboratories, Hercules, CA, USA) and gene expression was quantified by using the delta C(T) method by normalizing to the expression of *β-actin*.

Statistical Methods

Continuous variables are summarized as means with ranges or bootstrapped 95% confidence intervals (CI), or medians with interquartile ranges (IQR). Values measured at study entry and after 3 months were compared using Wilcoxon signed rank test. Correlation between variables was assessed by Spearman rank correlation. No adjustment was made for multiple testing and p-values equal or less than 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS version 25 (IBM, Armonk, NY, USA) and Stata version 15 (StataCorp LLC, College Station, TX, USA).

RESULTS

Patients

Eighteen patients, 9 from each outpatient clinic, were recruited to the study. Of these, one patient did not start tofacitinib treatment and for another patient flow cytometry results could not be obtained at baseline visit due to a technical problem with the flow cytometer. The final study population therefore consisted of 16 patients, who continued tofacitinib treatment until follow-up visit and had complete data sets from both visits.

Characteristics of the patient cohort at baseline visit are presented in **Table 1**. The background csDMARD therapy of each patient is described in detail in **Supplementary Table 4**. A total of 12 patients (75%) received methotrexate as part of their csDMARD regimen (6 with triple, 4 with double, and 2 with single csDMARD therapy).

Clinical Response to Tofacitinib

Tofacitinib with background csDMARD treatment significantly decreased the activity of RA during 3-month treatment, as defined both by clinical measures of activity, as well as by patient-reported outcomes (**Table 2**). Median DAS28 score decreased from 4.4 to 2.6. Nine patients (56%) were in DAS28 remission at the 3-month visit. Four patients had low, two patients moderate and one patient high

TABLE 1 | Characteristics of the patients (n=16).

Female sex, n (%)	11 (69%)
Age, years, mean (range)	58.4 (36.6-72.9)
Disease duration, years, mean (range)	9.6 (0.5-48.0)
Rheumatoid factor positive, n (%)	11 (69%)
CCP-antibody positive, n (%)	12 (75%)
Erosive disease, n (%)	7 (44%)
Disease activity (DAS28), n (%)	
Moderate	13 (81%)
High	3 (19%)
csDMARD regimen, n (%)	
Triple	6 (37%)
Double	7 (44%)
Single	3 (19%)
Low-dose prednisolone, n (%)	8 (50%)

CCP, cyclic citrullinated peptide; DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; n, number of patients.

disease activity according to DAS28 following the treatment with tofacitinib and csDMARDs.

Safety laboratory tests remained within acceptable range (**Table 2**) and there were no serious adverse events during the study.

Tofacitinib Suppresses Both Constitutive and Cytokine-Induced STAT Phosphorylation *In Vivo* in Cytokine and Cell Type Specific Matter

Constitutive and *in vitro* cytokine-induced STAT phosphorylation in monocytes, T cells and B cells was measured using multi-color flow cytometry at baseline and three-month visits. Based on the pharmacokinetic profile of tofacitinib (29), our results describe the maximal *in vivo* effect of tofacitinib, as the blood samples at three-month visit were collected shortly after morning tofacitinib dose. The flow cytometric assay was performed using fresh blood without cell isolation, and thus is likely to reflect STAT phosphorylation

in vivo. Cytokine-induced STAT phosphorylation was studied using selected cytokine or cytokines for each pSTAT (**Figure 2**).

Tofacitinib, in csDMARD background, decreased significantly constitutive phosphorylation of STATs (**Figure 2**). The decrease in constitutive phosphorylation was most consistent for STAT3 (shown in all cell types studied). In addition, constitutive phosphorylation of STAT1, STAT4 and STAT5 was also downregulated by tofacitinib in a cell type specific manner. Using similar methodology as in the current study, we have previously shown that constitutive pSTAT1, pSTAT3 and pSTAT5 are increased in circulating T cells and pSTAT3 in monocytes of RA patients compared to healthy volunteers (7, 10). In order to gain some insight into what extent these leukocyte signaling aberrations are normalized by tofacitinib treatment, we compared the differences in constitutive STAT phosphorylation between controls and RA patients (historical data) to those between tofacitinib-treated and untreated RA patients derived from the current study (**Supplementary Table 5**). This comparison suggests that the elevated pSTAT1, pSTAT3 and pSTAT5 levels in patients with RA are reversed to a significant degree by treatment with tofacitinib.

Cytokine-induced STAT phosphorylations were significantly decreased by tofacitinib in all studied leukocyte subtypes (**Figure 2**). In order to investigate the inhibitory potency of tofacitinib on STAT activation induced by different cytokines more closely, mean inhibition percentages between 3-month and baseline visits were calculated (**Table 3**). Depending on the cytokine and cell population, the mean percentage inhibition of STAT phosphorylation by tofacitinib treatment ranged from 10% to 73%. At least 50% inhibition was observed with STAT phosphorylations induced by IFN-α and common-γ-chain cytokines IL-2, IL-4, IL-15 and IL-21 in CD4$^+$ T cells, by IL-2, IL-4 and IL-21 in CD4$^-$ T cells and by IFN-γ in B cells. Lowest inhibition was demonstrated for IL-10 -induced STAT3 phosphorylation in monocytes. In general, monocyte responses were less sensitive to suppression by tofacitinib than those in CD4$^+$ T cells.

TABLE 2 | Comparison of clinical and laboratory parameters at baseline and after 3-month treatment with tofacitinib and csDMARDs.

	Before, median (IQR)	After 3 months, median (IQR)	p
Swollen joint count, 0-46	7 (6-9)	2 (0-3)	**<0.001**
Tender joint count, 0-46	11 (5-17)	1 (0-8)	**<0.001**
Swollen joint count, 0-28	5 (4-7)	1 (0-2)	**<0.001**
Tender joint count, 0-28	4 (2-10)	1 (0-3)	**<0.001**
General health, VAS, 0-100 mm	51 (37-65)	16 (7-29)	**0.001**
Pain, VAS, 0-100 mm	43 (22-64)	12 (5-38)	**0.002**
Physician's assessment, VAS, 0-100 mm	35 (31-46)	13 (11-18)	**<0.001**
HAQ disability index, 0-3	0.813 (0.625-1.253)	0.130 (0-0.813)	**0.011**
DAS28	4.4 (3.6-4.9)	2.6 (1.9-2.9)	**<0.001**
Plasma C-reactive protein, mg/l	5 (3-17)	3 (3-4)	**0.042**
Blood hemoglobin, g/l	129 (126-140)	132 (126-145)	0.775
Blood leukocyte count, ×10^9/l	8.3 (6.1-9.1)	5.3 (4.3-6.9)	**0.003**
Blood neutrophil count, ×10^9/l	5.32 (4.02-6.34)	2.88 (2.32-3.91)	**0.003**
Blood lymphocyte count, ×10^9/l	1.40 (1.07-1.94)	1.41 (1.20-1.83)	0.959
Blood platelet count, ×10^9/l	297 (274-334)	281 (225-302)	**<0.001**
Plasma alanine aminotransferase, U/l	20 (15-26)	21 (19-28)	0.224
Plasma creatinine, µmol/l	63 (56-83)	68 (56-86)	0.615

p-values are calculated using Wilcoxon test and shown in bold when p ≤ 0.05.
DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; IQR, interquartile range; VAS, visual analogue scale.
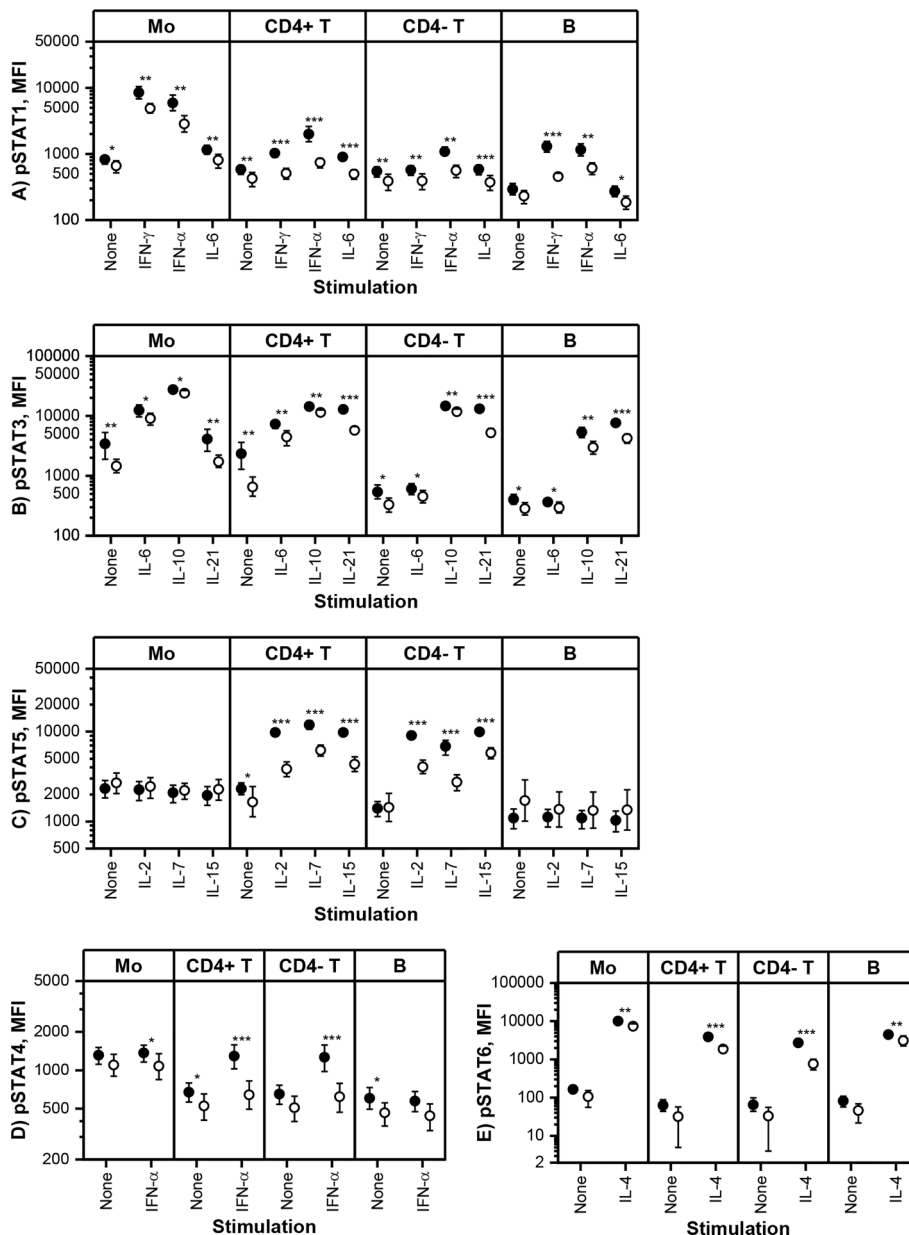
FIGURE 2 | Means and 95% confidence intervals of **(A)** phosphorylated STAT1 (pSTAT1), **(B)** pSTAT3, **(C)** pSTAT5, **(D)** pSTAT4 and **(E)** pSTAT6 median fluorescence intensities (MFI) in monocytes (Mo), CD4+ T cells (CD4+ T), CD4- T cells (CD4- T) and CD20+ B cells (B) at baseline (filled symbols) and after 3-month treatment with tofacitinib and csDMARDs (open symbols). Note the logarithmic scale of the Y-axis. p-values comparing 0 and 3 months are calculated by Wilcoxon test. Significant differences are marked with an asterisk: *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. csDMARD, conventional systemic disease-modifying antirheumatic drug; (p)STAT, (phosphorylated) signal transducer and activator of transcription.

Tofacitinib Downregulates the mRNA Expression of JAK-STAT Signaling Pathway Components

We also measured the total amount of STAT1 and STAT3 proteins in monocytes, T cells and B cells by flow cytometry. Tofacitinib did not cause significant changes in total STAT1 and STAT3 protein levels (**Figures 3A, B**).

The effect of tofacitinib on the mRNA levels of JAK-STAT pathway components and inhibitors in PBMCs was studied using qPCR. Significant, but rather modest, decreases were observed in the expression of *STAT3, STAT4, STAT5A, JAK1, JAK3* by tofacitinib, whereas there was no effect on *STAT1, STAT5B, STAT6, JAK2* and *TYK2* expression (**Figures 3C, D**). In contrast, the expression of all *SOCS*s studied was significantly

TABLE 3 | Mean (95% confidence interval) percentage inhibition of cytokine-induced STAT phosphorylation by tofacitinib in different cell populations at three months compared to baseline.

Cytokine	JAKs	STAT	Percentage inhibition in different cell types			
			Monocytes	CD4+ T cells	CD4- T cells	B cells
IFN-γ	JAK1/2	pSTAT1	34 (22;45)	49 (40;58)		**58** (45;68)
IFN-α	JAK1/TYK2	pSTAT1	34 (7;54)	**50** (35;63)	43 (31;56)	30 (0.4;52)
IFN-α	JAK1/TYK2	pSTAT4		45 (34;56)	45 (34;56)	
IL-2	JAK1/3	pSTAT5		**60** (50;69)	**54** (44;64)	
IL-4	JAK1/3	pSTAT6	25 (14;36)	**53** (44;61)	**73** (65;80)	25 (3;52)
IL-7	JAK1/3	pSTAT5		47 (37;57)	47 (25;63)	
IL-15	JAK1/3	pSTAT5		**55** (43;62)	40 (29;50)	
IL-21	JAK1/3	pSTAT3		**55** (47;62)	**60** (53;67)	42 (33;53)
IL-6	JAK1/2/TYK2	pSTAT1	29 (18;39)	41 (31;51)		
IL-6	JAK1/2/TYK2	pSTAT3	12 (-30;45)	42 (27;57)	22 (9;35)	
IL-10	JAK1/TYK2	pSTAT3	10 (-1;21)	19 (12;26)	19(10;27)	43 (28;56)

Results with percentages ≥ 50 are shown in bold. Spaces left empty denote the cases in which the stimulated phosphorylation level does not differ from the constitutive level. JAK proteins involved in each cytokine signaling pathway studied are presented in the Table.
IFN, interferon; IL, interleukin; JAK, Janus kinase; (p)STAT, (phosphorylated) signal transducer and activator of transcription; TYK2, tyrosine kinase 2.
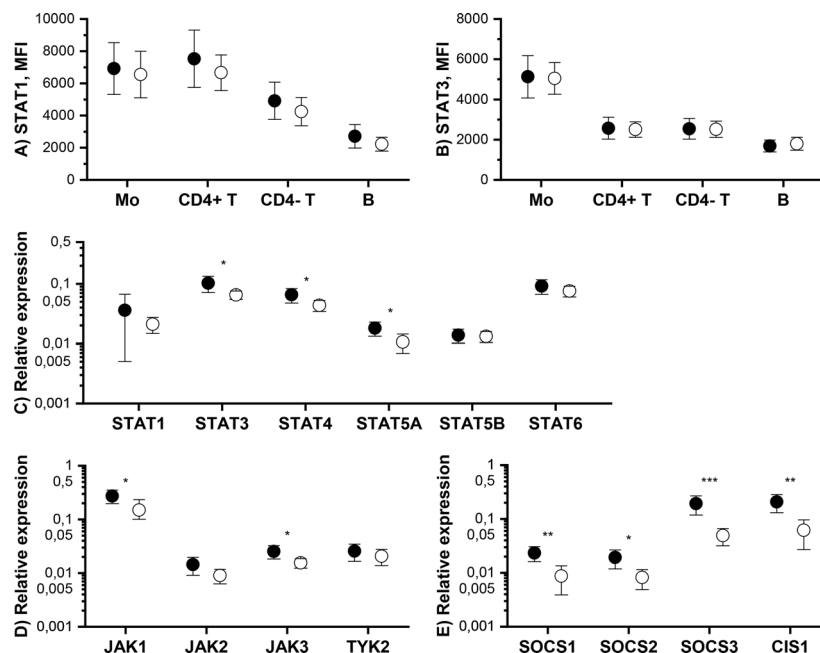
FIGURE 3 | Means and 95% confidence intervals of total **(A)** STAT1 and **(B)** STAT3 protein expression level in monocytes (Mo), CD4+ T cells (CD4+ T), CD4- T cells (CD4- T) and B cells and mRNA expression levels of **(C)** STAT, **(D)** JAK and **(E)** SOCS genes in PBMCs at baseline (filled symbols) and after 3-month treatment with tofacitinib and csDMARDs (open symbols). p-values comparing 0 and 3 months are calculated by Wilcoxon test. Significant differences are marked with an asterisk: *p ≤ 0.05, ** p ≤ 0.01, p***≤ 0.001. CIS1, cytokine-inducible SH2 domain-containing protein; csDMARD, conventional systemic disease-modifying antirheumatic drug; PBMC, peripheral blood mononuclear cell; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

downregulated during treatment with tofacitinib and csDMARDS (**Figure 3E**).

Baseline STAT Phosphorylation in Monocytes and T Cells and Expression of SOCS3 in PBMCs Correlate With Treatment Response

To study whether baseline signaling profile is associated with treatment response to tofacitinib, correlation coefficients were

calculated between baseline pSTATs, total STAT1 and STAT3 and the change from baseline in DAS28 after 3 months of treatment with tofacitinib and csDMARDs (**Table 4**).

Constitutive pSTAT1 and pSTAT3 in monocytes and constitutive pSTAT1, pSTAT3 and pSTAT5 in CD4+ T cells correlated positively with treatment response (**Supplementary Figures 1A–D, G**), while constitutive STAT4 phosphorylation in monocytes or in CD4- T cells correlated negatively with treatment response (**Supplementary Figures 1E, F**). In addition, IL-6-

TABLE 4 | Correlation of baseline pSTAT levels and total STAT1 and STAT3 expression levels with improvement in DAS28 during 3-month treatment with tofacitinib and csDMARDs.

Molecule	Stim.	Cell type							
		Monocytes		CD4+ T cells		CD4- T cells		B cells	
		r	p	r	p	r	p	r	p
pSTAT1	None	**0.591**	**0.016**	**0.603**	**0.013**	0.391	0.134	0.280	0.294
pSTAT1	IFN-γ	0.379	0.147	**0.532**	**0.034**			0.062	0.820
pSTAT1	IFN-α	-0.079	0.770	-0.150	0.579	-0.088	0.745	0.038	0.888
pSTAT1	IL-6	**0.556**	**0.025**	0.282	0.289				
pSTAT3	None	**0.635**	**0.008**	**0.732**	**0.001**	0.424	0.102	0.426	0.099
pSTAT3	IL-6	0.385	0.141	**0.621**	**0.010**	0.126	0.641		
pSTAT3	IL-10	0.253	0.345	0.224	0.405	0.185	0.492	0.224	0.405
pSTAT3	IL-21			0.115	0.672	-0.009	0.974	0.085	0.753
pSTAT4	None	**-0.506**	**0.046**	-0.491	0.053	**-0.518**	**0.040**	-0.344	0.192
pSTAT4	IFN-α			**-0.568**	**0.022**	**-0.638**	**0.008**		
pSTAT5	None	0.350	0.184	**0.568**	**0.022**	0.271	0.311	0.276	0.300
pSTAT5	IL-2			0.147	0.587	0.159	0.557		
pSTAT5	IL-7			0.026	0.922	-0.147	0.587		
pSTAT5	IL-15			0.162	0.549	0.147	0.587		
pSTAT6	None	-0.175	0.517	-0.130	0.633	-0.219	0.414	0.218	0.418
pSTAT6	IL-4	-0.082	0.762	-0.300	0.259	-0.412	0.113	-0.279	0.295
STAT1	None	-0.291	0.274	-0.356	0.176	-0.488	0.055	-0.212	0.431
STAT3	None	-0.307	0.265	-0.354	0.196	-0.479	0.071	-0.432	0.108

Spearman correlation coefficients (r) are used. Results with p-values ≤0.05 are shown in bold. Spaces left empty denote the cases in which the stimulated phosphorylation level does not differ from the constitutive level.
DAS28, composite Disease Activity Score for 28 joints based on the C-reactive protein level (DAS28-4[CRP]); csDMARD, conventional systemic disease-modifying antirheumatic drug; IFN, interferon; IL, interleukin; (p)STAT, (phosphorylated) signal transducer and activator of transcription; stim., stimulation.

stimulated pSTAT1 levels in monocytes, IFN-γ-stimulated pSTAT1 levels in CD4+ T cells and IL-6-stimulated pSTAT3 in CD4+ T cells correlated positively with treatment response. IFN-α-stimulated pSTAT4 in T cells correlated negatively with treatment response.

In addition, we studied the correlations between baseline demographic, clinical or laboratory variables and response to treatment with tofacitinib and csDMARDs. DAS28 (r=0.549; p=0.028) and CRP level (r=0.554; p=0.026) significantly associated with treatment response while the other parameters showed no correlation (**Supplementary Table 6**).

The association between the decrease in pSTAT or STAT levels during treatment and tofacitinib treatment response was also investigated. Decrease in constitutive STAT3 phosphorylation in monocytes (r =0.600, p=0.014) and in CD4+ T cells (r=0.682, p=0.004) correlated positively with treatment response (**Supplementary Table 7**).

Finally, correlation coefficients were calculated between baseline *STAT*, *JAK* and *SOCS* mRNA levels in PBMC, or the change in mRNA expression levels, and the tofacitinib treatment response (**Supplementary Table 8**). Baseline *SOCS3* levels correlated positively with treatment response (r = 0.532; p = 0.034).

DISCUSSION

In this study we show that in patients with chronic csDMARD-unresponsive rheumatoid arthritis, tofacitinib suppresses multiple JAK-STAT pathways *in vivo* by decreasing both constitutive and cytokine-induced STAT phosphorylation in circulating leukocytes.

However, the level of suppression by tofacitinib depends on the cytokine and cell type studied and is somewhat different from that reported in previous *in vitro* studies. Moreover, tofacitinib inhibits mRNA expression of several JAK-STAT pathway components and inhibitory proteins. We also show that baseline pSTAT levels in monocytes and T cells and *SOCS3* level in PBMCs correlate with treatment response.

The selectivity of JAK inhibitors is currently a major subject of interest. Recently, three *in vitro* articles comparing the potencies of several JAK inhibitors towards different cytokine signaling pathways in PBMC or blood of healthy donors have been published (17, 18, 26). Even though *in vitro* cellular modelling is a useful tool and allows direct comparison between different JAK inhibitors, the *in vitro* results do not necessarily demonstrate the actual effects of JAK inhibitors *in vivo*. Indeed, our current results reveal novel information on the effects of tofacitinib on different JAK-STAT pathways *in vivo*.

Regarding the cytokine pathways that are preferentially suppressed by tofacitinib, both *in vitro* studies (17, 18, 26) and our current study show that tofacitinib potently suppresses JAK1/JAK3 -mediated signaling induced by the common-γ-chain cytokines IL-2, IL-4, IL-15 and IL-21. Our results also indicate that both IFN-α and IFN-γ responses were suppressed by tofacitinib to almost the same extent as responses induced by the common-γ-chain cytokines, while *in vitro* studies have demonstrated variable effects of tofacitinib on IFN signaling. Regarding that interferons are crucial mediators of antiviral responses, and that the risk of herpes zoster infection is significantly increased upon JAK inhibitor use (30), the significant inhibitory effect of tofacitinib on interferon-

induced STAT1 signaling we revealed *in vivo* provides one possible mechanism for the herpes zoster infection susceptibility among tofacitinib users.

Our current *in vivo* results showed at least three inhibitory characteristics of tofacitinib that differed from the results obtained *in vitro*. First difference relates to the magnitude of inhibition of cytokine-induced STAT phosphorylation by tofacitinib. For example, the estimated daily average inhibition percentages of common-γ-chain cytokine (IL-2, IL-4, IL-15 and IL-21) responses in CD4^{+} T cells (18, 26) or lymphocytes (17) were comparable to the maximal inhibition percentages that we demonstrate in CD4^{+} T cells for these pathways *in vivo* (between 47 to 60%). Although the average and maximal inhibition percentages are not directly comparable, the current data nevertheless suggest that the *in vitro* studies may overestimate the inhibitory potential of tofacitinib on JAK-STAT pathway activation in RA patients *in vivo*. Our results also show that even after three months of tofacitinib treatment, cytokines can still induce STAT phosphorylation that exceeds the constitutive phosphorylation level. Therefore, the current results, although demonstrating a significant inhibitory effect of tofacitinib on constitutive and cytokine-induced STAT phosphorylation, further support the prevailing idea that oral dosing of JAK inhibitors, such as tofacitinib, allows only partial and reversible inhibition of JAK-STAT pathways (26, 31).

Second, the inhibitory effects of tofacitinib on IL-4, IL-6 and IL-10 -induced STAT phosphorylations in monocytes were lower *in vivo* than those described *in vitro* (17, 18, 26). IL-10 induced STAT3 phosphorylation demonstrated the lowest (10%) inhibition by tofacitinib in the current study. This indicates that the potent anti-inflammatory actions that IL-10 has on monocytes and macrophages (32) may not be efficiently suppressed by tofacitinib *in vivo*. In addition, regarding the potent anti-inflammatory and anti-arthritic effects of the IL-4/STAT6 pathway (33), the only modest *in vivo* potency of tofacitinib on IL-4-stimulated STAT6 phosphorylation, together with its weak effect on IL-10-stimulated STAT3 phosphorylation, may be advantageous features for the clinical efficacy of tofacitinib. It is also of note that in the present study, constitutive phosphorylation of STAT6, unlike that of the other STATs, was not decreased in any leukocyte subtype studied during 3-month tofacitinib treatment.

Third, *in vitro* data suggest that IL-6-induced STAT1 phosphorylation is more strongly inhibited by tofacitinib than IL-6-induced STAT3 phosphorylation in T cells (17, 18), whereas we demonstrate comparable inhibition of both IL-6-induced STAT pathways in CD4^{+} T cells of RA patients *in vivo*. Actually, regarding our previous finding that constitutive STAT3 phosphorylation is common in circulating CD4^{+} T cells in RA and associates with disease activity (8), one mechanism implementing the efficacy of tofacitinib in treating RA might be the relatively good inhibitory effect on CD4^{+} T cell STAT3 phosphorylation *in vivo*.

There are several possible explanations for the above-mentioned differences. We used cytokine concentrations that are likely to induce maximal STAT phosphorylation, whereas lower cytokine concentrations were generally used in *in vitro* studies. *In vivo* conditions also include more variables than *in vitro* studies; for example, in our study RA patients were also treated with

csDMARDs, which may influence the magnitude of the inhibitory responses seen with tofacitinib, and furthermore, the conditions in which circulating leukocytes sense tofacitinib after oral administration are surely different from those in *in vitro* incubations. Finally, during three-month treatment, tofacitinib is likely to cause long-term effects that influence the inhibitory potential of tofacitinib and its selectivity towards different cytokine pathways and thus, results obtained by studying samples of healthy volunteers in *in vitro* studies may in principle be somewhat different from ours. In fact, we demonstrate that tofacitinib treatment suppresses the mRNA expression of certain components of the JAK-STAT pathway. In particular, the expression of inhibitory molecules *SOCS*s were downregulated, which may affect the overall inhibitory state of JAK-STAT pathways and that way also influence tofacitinib's inhibitory potential *in vivo*.

The effect of tofacitinib on mRNA expression of *JAK*s, *STAT*s and *SOCS*s has not been extensively studied before. As cytokines are well-characterized inducers of *SOCS1-3* and *CIS1* expression (34), the observed decrease in their mRNA expression in the current study seems rational upon JAK inhibition. Decrease in *SOCS3* expression was the most prominent finding, and baseline *SOCS3* levels also correlated with the treatment response. This is an interesting observation regarding that STAT3-regulated *SOCS3* expression in CD4^{+} T cells has been shown to be elevated in a cohort of 161 treatment-naïve early arthritis patients (35) and in PBMCs from patients with active RA (36). Our data suggests that tofacitinib treatment also slightly but significantly decreases *STAT3*, *STAT4*, *STAT5A*, *JAK1* and *JAK3* expression. However, as STAT3 protein levels were not repressed after the 3-month tofacitinib treatment, the mechanistic significance of the changes in STAT mRNA expression remains elusive. As tofacitinib has been shown to decrease expression of extracellular proteases, such as matrix metalloprotease 1 in rheumatoid arthritis synovial fibroblasts (37), it is possible that the inhibitory effect of tofacitinib extends also to intracellular protein degradation, such as the ubiquitin-proteasome system. This could explain the unchanged STAT3 protein level.

In order to estimate how tofacitinib-specific the observed decrease in constitutive and cytokine-induced phosphorylation of STATs is, we compared the current results to our previous results on csDMARD-unresponsive RA patients treated with biologic DMARD (11). During biological DMARD treatment, IL-4 -induced pSTAT6 was downregulated. Interestingly, the effect was clearly weaker than that shown with tofacitinib in the current study. Strikingly, neither constitutive nor IFN-γ-stimulated pSTAT1 showed significant changes during biological DMARD treatment, while the correspondent pSTAT1 levels fell significantly by tofacitinib use. For example, tofacitinib downregulated IFN-γ-stimulated pSTAT1 in monocytes by 34% and in CD4^{+} T cells by 49%. Although results from different studies are not directly comparable, the data so far suggest that decrease in STAT phosphorylation is not a general effect achieved by any RA medication.

The association between baseline STAT phosphorylations and treatment response to tofacitinib was also examined. Baseline constitutive pSTAT1 and pSTAT3 levels in monocytes and CD4^{+} T cells, and pSTAT5 levels in CD4^{+} T cells correlated positively with the response. The strongest correlation was seen

with pSTAT3. The magnitude of decrease in pSTAT3 levels following tofacitinib treatment also correlated positively with treatment response. As we and others have previously shown that constitutive pSTAT1, pSTAT3 and pSTAT5 levels in T cells and pSTAT3 levels in monocytes are elevated in RA patients (7, 9, 10), tofacitinib obviously targets several RA-associated leukocyte signaling aberrations successfully. The comparison of STAT phosphorylation levels between controls and RA patients (historical data) to those between tofacitinib-treated and untreated RA patients derived from the current study suggests that the increased constitutive phosphorylation of STATs in patients with RA is reversed to a significant degree *in vivo* by tofacitinib. However, as healthy controls were not included in the current study, it remains unresolved whether the patients' STAT phosphorylation levels reached those of healthy individuals.

The only inverse correlation we found between baseline pSTAT levels and treatment response concerned STAT4. In this context it may be noteworthy that STAT4 is able to cause sustained expression of genes that increase sensitivity to IL-18 (38). IL-18 signaling does not take place *via* JAK-STAT pathways and hence, is not directly affected by tofacitinib. Both IL-18 receptor and STAT4 deficiency have suppressed the severity of arthritis in a murine model of RA (39, 40). Furthermore, STAT4 represses the genes of the Th2 cytokines IL-5 and IL-13 (41), both of which have been associated with arthritis-limiting capacity and decreased progression to fully established RA (33). Hence, STAT4 phosphorylation measured in the patients' leukocytes at baseline may be associated with immunological features that are not so easily amended by tofacitinib. It is also notable that genetic variation of STAT4 has been associated with the risk of autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis (42). It remains to be elucidated whether STAT4 variants are associated with STAT4 phosphorylation levels and RA patients' response to tofacitinib.

The effect of tofacitinib treatment on the pathobiology of rheumatoid synovium has been studied by Boyle et al. using immunohistochemistry (43). Their results showed a positive correlation between 4-month clinical improvement and reductions in STAT1 and STAT3 phosphorylation at day 28 (43). However, unlike ours, their results did not show associations between baseline STAT phosphorylation levels and the clinical response. This discrepancy might be due to methodological differences between the two studies, but it is also possible that constitutive and cytokine-stimulated pSTAT levels in circulating leukocytes, rather than in synovial cells, represent a more sensitive sensor of the overall immunological state. Nevertheless, further studies are needed to show whether STAT phosphorylation or *SOCS3* expression could be used as a biomarker to predict clinical response to tofacitinib treatment.

The strengths of the current study are: 1) prospective study on well-characterized RA patients in whom multiple JAK-STAT signaling pathways were analyzed close to *in vivo* conditions before and three months after starting tofacitinib therapy; 2) the use of whole blood flow cytometric assay, which minimizes inappropriate cell signaling pathway activation due to sample handling. The limitation of the study is that the relatively small patient cohort enables only predictive conclusions on the clinical significance of the observed association between baseline immunological parameters and treatment response. Even though it needs to be confirmed in further studies if the baseline JAK-STAT signaling profile has prognostic value for tofacitinib treatment responses, this study significantly adds to our understanding about the mechanisms of tofacitinib function in RA patients receiving this treatment.

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 National Committee on Medical Research Ethics (TUKIJA) and Finnish Medicines Agency Fimea. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.738481/full#supplementary-material

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The Shared Mechanism and Candidate Drugs of Multiple Sclerosis and Sjögren's Syndrome Analyzed by Bioinformatics Based on GWAS and Transcriptome Data

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Objective: This study aimed to explore the shared mechanism and candidate drugs of multiple sclerosis (MS) and Sjögren's syndrome (SS).

Methods: MS- and SS-related susceptibility genes and differentially expressed genes (DEGs) were identified by bioinformatics analysis based on genome-wide association studies (GWAS) and transcriptome data from GWAS catalog and Gene Expression Omnibus (GEO) database. Pathway enrichment, Gene Ontology (GO) analysis, and protein–protein interaction analysis for susceptibility genes and DEGs were performed. The drugs targeting common pathways/genes were obtained through Comparative Toxicogenomics Database (CTD), DrugBank database, and Drug–Gene Interaction (DGI) Database. The target genes of approved/investigational drugs for MS and SS were obtained through DrugBank and compared with the common susceptibility genes.

Results: Based on GWAS data, we found 14 hub common susceptibility genes (*HLA-DRB1*, *HLA-DRA*, *STAT3*, *JAK1*, *HLA-B*, *HLA-DQA1*, *HLA-DQA2*, *HLA-DQB1*, *HLA-DRB5*, *HLA-DPA1*, *HLA-DPB1*, *TYK2*, *IL2RA*, and *MAPK1*), with 8 drugs targeting two or more than two genes, and 28 common susceptibility pathways, with 15 drugs targeting three or more than three pathways. Based on transcriptome data, we found 3 hub common DEGs (*STAT1*, *GATA3*, *PIK3CA*) with 3 drugs and 10 common risk pathways with 435 drugs. "JAK-STAT signaling pathway" was included in common susceptibility pathways and common risk pathways at the same time. There were 133 overlaps including JAK-STAT inhibitors between agents from GWAS and transcriptome data. Besides, we found that *IL2RA* and *HLA-DRB1*, identified as hub common susceptibility genes, were the targets of daclizumab and glatiramer that were used for MS, indicating that daclizumab and glatiramer may be therapeutic for SS.

Conclusion: We observed the shared mechanism of MS and SS, in which JAK-STAT signaling pathway played a vital role, which may be the genetic and molecular bases of comorbidity of MS with SS. Moreover, JAK-STAT inhibitors were potential therapies for MS and SS, especially for their comorbidity.

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) (1). The etiology and pathogenesis of MS are complex, in which autoimmune responses based on genetic susceptibility and environmental risk factors have a vital role. However, the initiation of autoimmune responses is not fully explained, which may be related to Epstein–Barr virus (EBV) antigen mimicking or epitope expansion in genetically susceptible individuals (2).

Comorbidity of MS with many other autoimmune diseases is found in our clinical work, including Sjögren's syndrome (SS). Epidemiological studies show that the prevalence of SS in MS patients is about 1% up to 16.7% in primary progressive MS patients, with the incidence of sicca syndrome (typical symptoms but not meeting the diagnostic criteria of SS) up to 10%, while the prevalence of SS in the general population is only 0.06%, indicating that the prevalence of SS in MS patients is higher than that in the general population (3). On the other hand, SS also involves CNS with a 5.8%–38% probability, and 52%–80% of these conditions occur before SS diagnosis (3).

SS is characterized by exocrinopathy and specific anti-SSA/SSB antibodies, resulting in dryness of the mouth and eyes, fatigue, and joint pain, can also involve CNS (4). The etiology and pathogenesis of SS are not clear. Both MS and SS are associated with the same environmental factors including EBV and cytomegalovirus (CMV) infection, vitamin D deficiency, and smoking (1, 5, 6). In addition, SS is also associated with autoimmune responses mainly mediated by T and B cells, which may be activated by abnormal mucosal epithelial cells stimulated by herpesvirus such as EBV (4). T cells play a major role in the early periods of SS, especially CD4$^+$ T cells while B cells contribute more in later progression (5).

Based on the epidemiological correlation between MS and SS and the similarities in environmental factors and pathogenesis, we speculated that there may be common genetic susceptibility factors and pathogenic pathways and then explored whether the therapeutic agents of these two diseases can provide reference for each other. Therefore, based on the available genome-wide association studies (GWAS) and transcriptome data, we analyzed the shared genes and pathways between MS and SS from different levels of susceptibility genes and differentially expressed genes (DEGs) and explored potential therapeutic drugs. Our study may be helpful in revealing the shared genetic etiology and pathogenesis underlying MS and SS. More importantly, these findings may provide clues to potential therapeutic strategies.

2 METHODS

2.1 Processing and Analysis of GWAS Data

2.1.1 GWAS Data Extraction and Identification of Susceptibility Genes

GWAS is a database that systematically summarizes the observed single-nucleotide polymorphism (SNP)–disease associations. GWAS data of MS and SS were obtained and downloaded from the GWAS Catalog (https://www.ebi.ac.uk/gwas/) up to January 2021 through searching "multiple sclerosis" and "Sjögren's syndrome," respectively, which include disease-related SNPs, reported genes, PubMed ID, sample size, ethical groups, *P*-value, study accession ID, and platform (**Supplementary Table S1** and **Supplementary Figure S1**) (6). For SNPs reported in GWAS, those with *P*-value ≤5.0 × 10^{-8} were included in our study. And the corresponding reported genes were collected and defined as the susceptibility genes of MS or SS. Hypergeometric test was performed to evaluate the significance of overlap of MS and SS susceptibility genes: phyper (k-1, M, N-M, n, lower.tail=F).

2.1.2 Enrichment Analysis and Functional Annotation of Common Susceptibility Genes

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the susceptibility genes was carried out by R package "clusterprofiler" (Version 4) (7, 8). Hypergeometric test was performed to evaluate the significance of pathway–pathway association: phyper (k-1, M, N-M, n, lower.tail=F). After common susceptibility pathways were obtained, susceptibility genes of MS/SS annotated to common susceptibility pathways were obtained and defined as common susceptibility genes in this study. And Gene Ontology (GO) annotation of the common susceptibility genes was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) (9, 10), including biological process (BP), cellular component (CC), and molecular function (MF). *P*-value <0.05 was determined as a significant margin for all analyses. Column chart was plotted by http://www.bioinformatics.com.cn, a free online platform for data analysis and visualization.

2.1.3 Pathway–Gene Network Construction

The common susceptibility pathway–gene network was constructed through Cytoscape (version 3.8.2) (11), and the correlation degree of gene nodes was calculated.

2.1.4 Protein–Protein Interaction Network Construction

The protein–protein interaction (PPI) network of common susceptibility genes was constructed using STRING (https://cn.

string-db.org/, version 11.5) and visualized by Cytoscape, and the correlation degree of nodes was calculated (12).

2.2 Processing and Analysis of Transcriptome Data

2.2.1 Transcriptome Data Extraction and Identification of Differentially Expressed Genes

Transcriptome data of MS and SS were obtained from the Gene Expression Omnibus (GEO) database (13). We evaluated two datasets with accession IDs GSE78244 and GSE117935 for MS and another two with GSE94510 and GSE135809 for SS. These four datasets are microarray gene expression data. GSE78244 dataset contains the data of $CD4^+$ T cells from 14 MS and 14 healthy control subjects, while GSE117935 contains those of B cells from 10 MS and 10 healthy control subjects, GSE94510 contains those of $CD4^+$ T cells from 6 SS and 6 healthy control subjects, and GSE135809 contains those of B cells from 6 SS and 6 healthy control subjects. The datasets were normalized by log2 transformation and quantile standardization using R package "Limma" (14). Significant DEGs with P-value <0.05 and fold change ≥1.2 were obtained by R, and common DEGs were identified by intersection.

2.2.2 Enrichment Analysis and Functional Annotation of Common Differentially Expressed Genes

GO annotation of common DEGs was performed by DAVID (the same as above). The KEGG pathway enrichment analysis of the DEGs was carried out by R package "clusterprofiler" (the same as above). Significant risk pathways of MS and SS and common risk pathways were obtained.

2.2.3 Protein–Protein Interaction Network Construction

The PPI network of common DEGs was constructed using STRING (the same as above).

2.3 Identification of Candidate Drugs for Multiple Sclerosis and Sjögren's Syndrome

2.3.1 Identification of Drugs Targeting Common Pathways/Genes

Drugs targeting common susceptibility pathways from GWAS data and common risk pathways from transcriptome data were identified using the Comparative Toxicogenomics Database (CTD) (version 16438) (15). Drugs targeting common susceptibility genes from GWAS data and common DEGs from transcriptome data were identified using DrugBank database (version 5.1.8) and Drug–Gene Interaction (DGI) Database (version 4.2.0) (16, 17). Then, drug–pathway and drug–gene interaction networks were constructed through Cytoscape. The drugs targeting three or more than three pathways and drugs targeting two or more than two genes were identified further. In addition, we compared the drugs identified from GWAS data and transcriptome data.

2.3.2 Analysis of Target Genes of Approved/ Investigational Drugs for Multiple Sclerosis and Sjögren's Syndrome

Approved drugs and investigational drugs for MS and SS were searched with "multiple sclerosis" and "Sjögren's syndrome" through the DrugBank database and *ClinicalTrials.gov* database (18). Investigational drugs were screened according to the following criteria. Inclusion criteria: ① The trial is in progress or has been completed; ② Definite drug composition. Exclusion criteria: ① Symptomatic drugs; ② Unclear composition; ③ Experiment terminated; ④ Non-drug treatment; ⑤ Approved drugs. Then, the target genes of drugs meeting the above criteria were obtained through the DrugBank database and compared with the common susceptibility genes found in this study.

The workflow of this study was shown in **Figure 1**.

3 RESULTS

3.1 Common Genes and Pathways of Multiple Sclerosis and Sjögren's Syndrome Based on GWAS Data

3.1.1 Identification of Susceptibility Genes and Enrichment Analysis

A total of 385 SNPs of MS and 19 SNPs of SS were obtained from GWAS data, as well as 260 and 22 susceptibility genes, respectively (**Supplementary Tables S1, S2** and **Supplementary Figures S1, S2**). Eleven overlapping genes with significance evaluated by hypergeometric test were found ($P = 9.02 \times 10^{-16}$). Ninety-three and 30 susceptibility pathways of MS and SS were obtained through KEGG pathway enrichment of susceptibility genes using R package "clusterprofiler" ($P < 0.05$). Twenty-eight common susceptibility pathways were obtained through Venn cross, and the classification was shown in **Figures 2A, B**. The first common susceptibility pathway according to the annotated gene number was the "Th1 and Th2 cell differentiation pathway" (**Figure 2C**). In order to evaluate the pathway–pathway association, the hypergeometric test was performed on 378 pathway–pathway pairs, of which 360 pairs were significant ($P < 0.05$) (**Supplementary Table S3**).

3.1.2 Identification and Analysis of Common Susceptibility Genes

Sixty common susceptibility genes of MS and SS were obtained (**Supplementary Table S4**). A total of 189 enriched GO terms were identified for common susceptibility genes (**Supplementary Table S5**). GO annotation showed that the most significant BP was the "interferon-gamma-mediated signaling pathway", CC was the "integral component of lumenal side of endoplasmic reticulum membrane", and MF was the "peptide antigen binding". The top 10 terms in the BP, CC, and MF categories were shown in **Figure 3A**.

From the common susceptibility pathway–gene network mapped by Cytoscape, 22 genes with degree ≥6 were identified (**Figure 3B**). Through PPI analysis and visualization by Cytoscape, 14 genes with degree ≥6 that were regarded as hub common susceptibility genes in our study (*HLA-DRB1, HLA-*
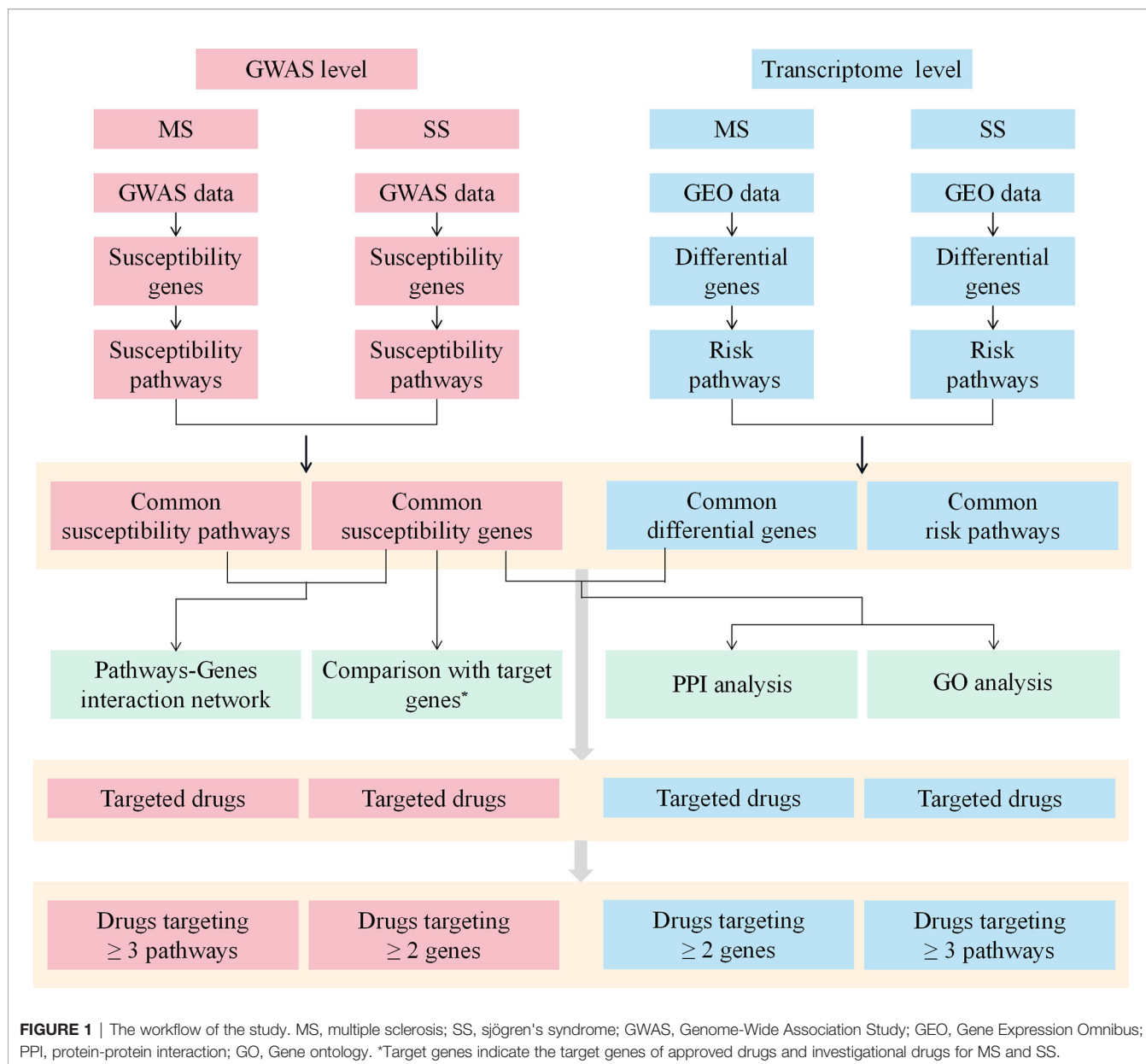
FIGURE 1 | The workflow of the study. MS, multiple sclerosis; SS, sjögren's syndrome; GWAS, Genome-Wide Association Study; GEO, Gene Expression Omnibus; PPI, protein-protein interaction; GO, Gene ontology. *Target genes indicate the target genes of approved drugs and investigational drugs for MS and SS.

DRA, *STAT3*, *JAK1*, *HLA-B*, *HLA-DQA1*, *HLA-DQA2*, *HLA-DQB1*, *HLA-DRB5*, *HLA-DPA1*, *HLA-DPB1*, *TYK2*, *IL2RA*, and *MAPK1*) were identified further (**Figure 3C**).

3.2 Common Genes and Pathways of Multiple Sclerosis and Sjögren's Syndrome Based on Transcriptome Data

MS and SS datasets were obtained from GEO database (**Table 1**). The list of DEGs, the fold change, and the associated *P*-values in CD4$^+$ T and B cells in MS and SS were shown in **Supplementary Table S6**. There were 109 common DEGs of MS and SS, including 18 DEGs in CD4$^+$ T cells and 91 DEGs in B cells. GO annotation showed that the most significant BP was "endothelial cell migration"; the CC with the most expressed genes was "nucleoplasm", but it did not reach significance ($P >$

0.05), and the most significant MF was "transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding" (**Figure 4A**). The PPI network with 45 nodes and 45 edges was constructed, and 3 hub common DEGs (*STAT1*, *PIK3CA*, and *GATA3*) with degree ≥6 were identified (**Figure 4B**). In terms of KEGG pathway enrichment, 35 and 74 significant risk pathways of MS and SS and 10 common risk pathways were obtained (**Table 2**).

3.3 Candidate Drugs for Multiple Sclerosis and Sjögren's Syndrome

3.3.1 Drugs Targeting Common Susceptibility Pathways/Genes Based on GWAS Data

A total of 295 drugs targeting common susceptibility pathways were found using CTD, of which antineoplastic drugs accounted for 41%;

FIGURE 2 | Common susceptibility pathways. (A) Venn diagram of common susceptibility pathways. (B) Classification of common susceptibility pathways. (C) Column chart of common susceptibility pathways. MS, multiple sclerosis; SS, sjögren's syndrome.
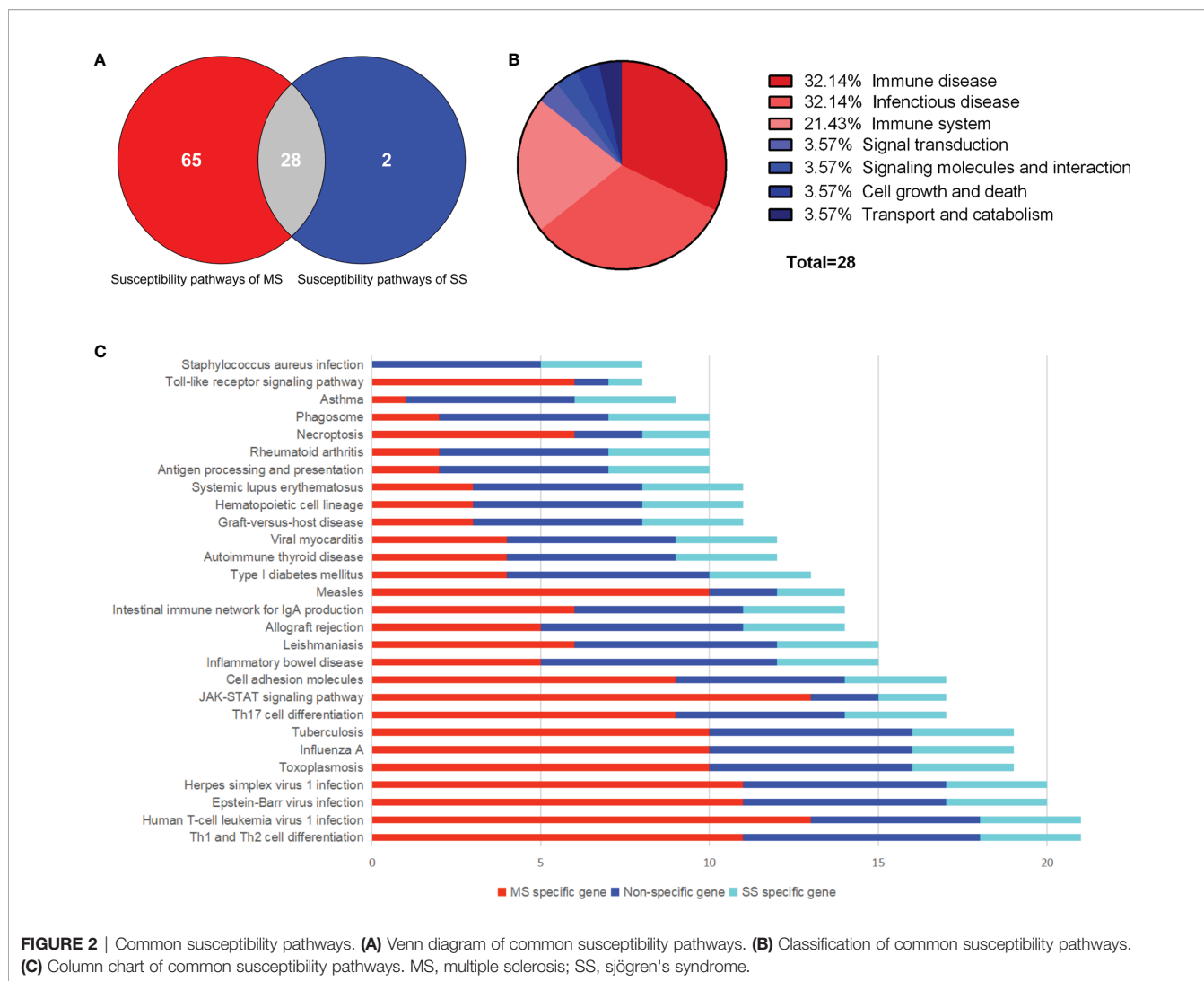
anti-inflammatory drugs, 15%; and immunodepressant drugs, 5%. There were 15 drugs targeting more than or equal to three pathways, such as filgotinib, delgocitinib, and brepocitinib (**Figure 5A** and **Table 3**). In addition, 168 drugs targeting common susceptibility genes were obtained through the DrugBank and DGI databases, of which 22 drugs targeted more than or equal to two common susceptibility genes (**Table 4**). And there were 68 drugs targeting hub common susceptibility genes (**Figure 5B**), of which 8 drugs targeted more than or equal to two common susceptibility genes, for example, filgotinib and tofacitinib (**Table 4**).

3.3.2 Drugs Targeting Common Risk Pathways/ Differentially Expressed Genes Based on Transcriptome Data

Drugs targeting common risk pathways were searched using CTD, and 435 drugs were found for 10 common risk pathways, of which no drug targeted more than or equal to three common risk pathways. In addition, 415 drugs targeting common DEGs were obtained using DrugBank and DGI databases, of which 12

drugs targeted more than or equal to two common DEGs, most of which were antineoplastic and immunodepressant drugs. There were three drugs targeting hub common DEGs including cisplatin, doxorubicin, and vincristine (**Table 5**).

3.3.3 Comparison of Candidate Drugs Identified From GWAS Data and Transcriptome Data

"JAK-STAT signaling pathway" and "influenza A" were included in common susceptibility pathways and common risk pathways at the same time. There were 295 drugs targeting common susceptibility pathways and 435 drugs targeting common risk pathways, with 105 overlaps that were mainly divided into 6 categories: hematopoietic drugs (26.7%), interleukin (IL) inhibitor (21.9%), interferon (INF) (17.1%), JAK-STAT inhibitor (15.2%), and recombinant IL (6.7%) (**Figure 6**). There were 168 drugs targeting common susceptibility genes and 415 drugs targeting common DEGs with 28 overlaps, of which 17 were antineoplastic drugs, such as cyclophosphamide, mercaptopurine, and thioguanine (**Supplementary Table S7**).
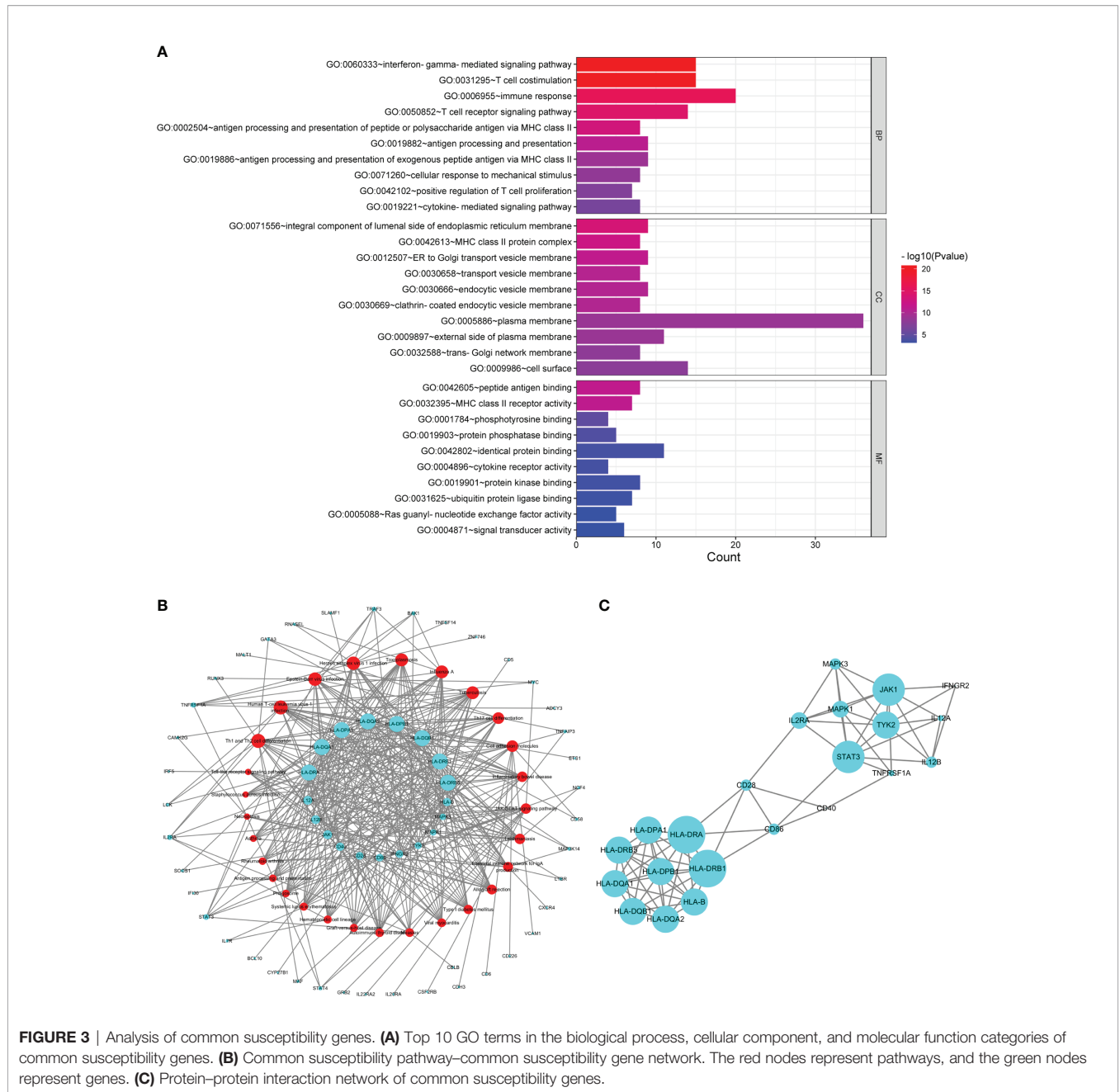
FIGURE 3 | Analysis of common susceptibility genes. **(A)** Top 10 GO terms in the biological process, cellular component, and molecular function categories of common susceptibility genes. **(B)** Common susceptibility pathway–common susceptibility gene network. The red nodes represent pathways, and the green nodes represent genes. **(C)** Protein–protein interaction network of common susceptibility genes.

3.3.4 Comparison of Target Genes of Approved/Investigational Drugs

Sixteen approved drugs with 39 target genes and 44 investigational drugs with 88 target genes of MS as well as 5 approved drugs with 9 target genes and 30 investigational drugs with 44 target genes of SS were obtained in this study (**Supplementary Table S8**). Through comparing hub common susceptibility genes and target genes of available drugs, we found that *IL2RA* and *HLA-DRB1* that were

TABLE 1 | Characteristics of transcriptome datasets.

ID	Disease	Sample	Sample size (control)	Platform ID	Date	DEG	Pathway
GSE78244	MS	CD4^{+} T cell	28 (14)	GPL17077	2016	714	35
GSE117935	MS	B cell	20 (10)	GPL5175	2018	293	
GSE94510	SS	CD4^{+} T cell	12 (6)	GPL570	2017	2,514	74
GSE135809	SS	B cell	12 (6)	GPL570	2019	7,373	
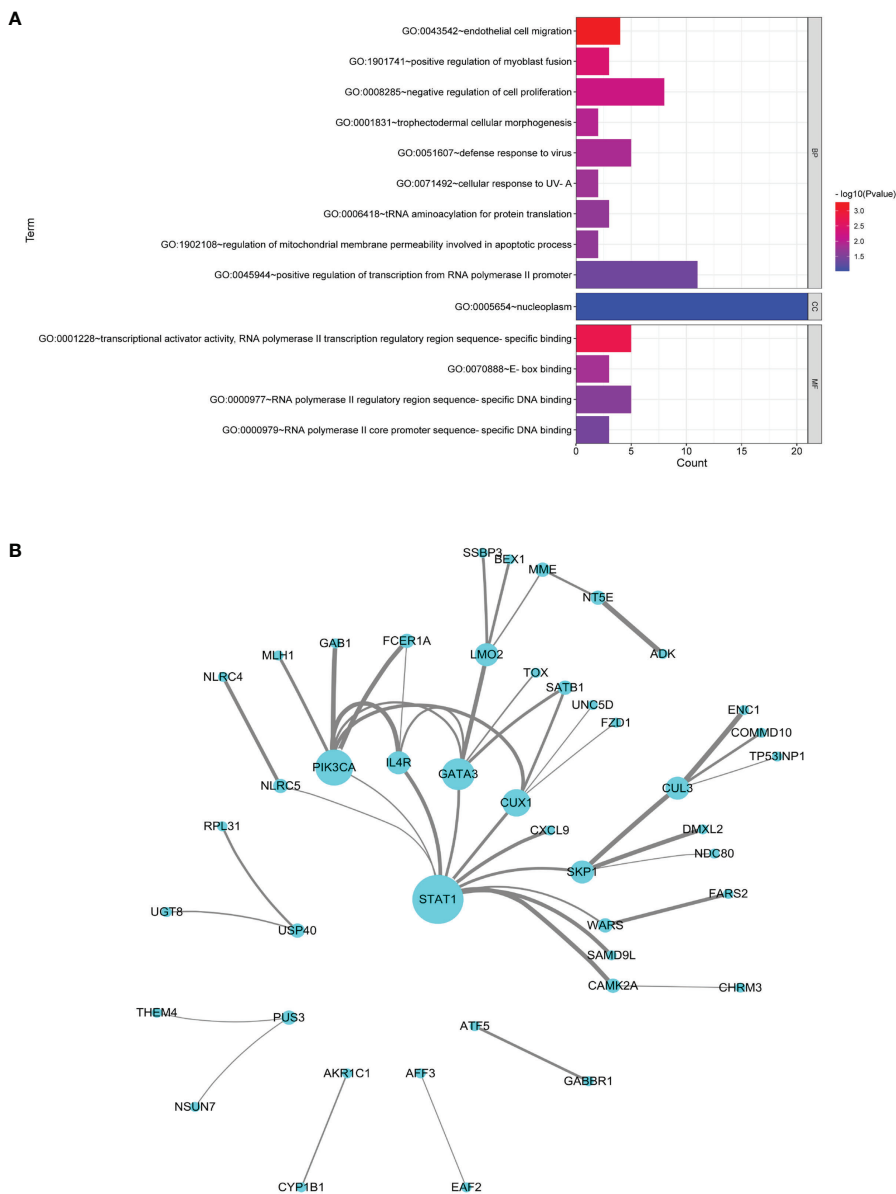
FIGURE 4 | Analysis of common DEGs. **(A)** GO terms in the biological process, cellular component, and molecular function categories of common DEGs. **(B)** Protein–protein interaction network of common DEGs. Nodes represent DEGs, and edges represent the combined score of PPI. The higher the combined score, the thicker the edge.

TABLE 2 | Common risk pathways of MS and SS.

KEGG_ID	KEGG_Term	P-value (MS)	P-value (SS)
hsa04630	JAK-STAT signaling pathway	0.019761431	0.002589011
hsa04614	Renin-angiotensin system	0.028086801	0.002850761
hsa04066	HIF-1 signaling pathway	0.001235913	0.003595271
hsa00380	Tryptophan metabolism	0.007088408	0.006280651
hsa00620	Pyruvate metabolism	0.047106391	0.007331886
hsa04061	Viral protein interaction with cytokine and cytokine receptor	0.008545436	0.007729449
hsa05164	Influenza A	0.027531183	0.014997505
hsa04550	Signaling pathways regulating pluripotency of stem cells	0.008786573	0.030629834
hsa04713	Circadian entrainment	0.007146748	0.032560706
hsa04728	Dopaminergic synapse	0.038600501	0.041333016
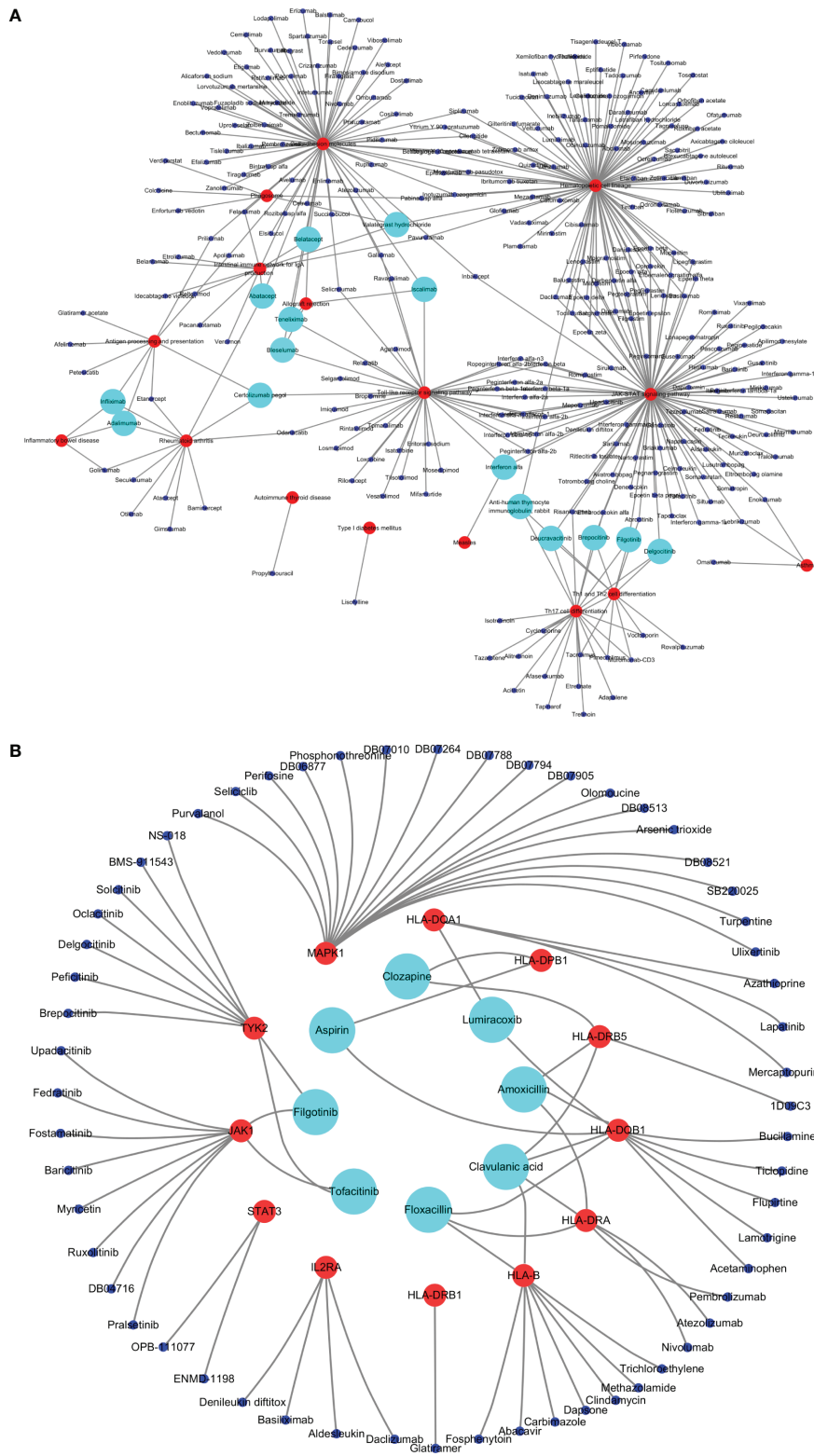
FIGURE 5 | Drugs targeting common susceptibility pathways/genes. (A) Drugs–common susceptibility pathways interaction network. The red nodes represent pathways, the blue nodes represent drugs, and the green nodes represent drugs targeting more than or equal to three pathways. (B) Drugs–hub common susceptibility genes interaction network. The red nodes represent genes, the blue nodes represent drugs, and the green nodes represent drugs targeting more than or equal to two genes.

TABLE 3 | Drugs targeting more than or equal to three common susceptibility pathways.

Drug	Target pathway	Effect
Abatacept	Intestinal immune network for IgA production	Selective costimulation modulator
	Cell adhesion molecules	Antirheumatic
	Rheumatoid arthritis	
Adalimumab	Inflammatory bowel disease	Anti-TNF-α antibody
	Antigen processing and presentation	Antirheumatic
	Rheumatoid arthritis	Anti-inflammatory
Anti-human thymocyte	Th1 and Th2 cell differentiation	Immunosuppressant
Immunoglobulin (rabbit)	Th17 cell differentiation	
	Hematopoietic cell lineage	
Belatacept	Allograft rejection	Selective costimulation modulator
	Intestinal immune network for IgA production	Immunosuppressant
	Cell adhesion molecules	Antirheumatic
Bleselumab	Allograft rejection	Anti-CD40 antibody
	Cell adhesion molecules	Immunosuppressant
	Toll-like receptor signaling pathway	
Brepocitinib	Th1 and Th2 cell differentiation	JAK1, TYK2 dual inhibitor
	Th17 cell differentiation	Anti-inflammatory
	JAK-STAT signaling pathway	
Certolizumab pegol	Antigen processing and presentation	Anti-TNF-α antibody
	Rheumatoid arthritis	Anti-inflammatory
	Toll-like receptor signaling pathway	Antirheumatic
Delgocitinib	Th1 and Th2 cell differentiation	JAK inhibitor
	Th17 cell differentiation	Anti-inflammatory
	JAK-STAT signaling pathway	Antipsoriatic
Deucravacitinib	Th1 and Th2 cell differentiation	TYK2 inhibitor
	Th17 cell differentiation	Anti-inflammatory
	JAK-STAT signaling pathway	
Filgotinib	Th1 and Th2 cell differentiation	JAK inhibitor
	Th17 cell differentiation	Anti-inflammatory
	JAK-STAT signaling pathway	
Infliximab	Inflammatory bowel disease	Anti-TNF-α antibody
	Antigen processing and presentation	Anti-inflammatory
	Rheumatoid arthritis	Antirheumatic
Interferon alfa	JAK-STAT signaling pathway	Antineoplastic
	Measles	Antiviral
	Toll-like receptor signaling pathway	Biological response modifier
Iscalimab	Allograft rejection	Anti-CD40 antibody
	Cell adhesion molecules	
	Toll-like receptor signaling pathway	
Teneliximab	Allograft rejection	Anti-CD40 antibody
	Cell adhesion molecules	Immunosuppressant
	Toll-like receptor signaling pathway	
Valategrast	Intestinal immune network for IgA production	ITGA4/ITGB1, ITGA4/ITGB7 dual inhibitor
hydrochloride	Cell adhesion molecules	Antiasthmatic
	Hematopoietic cell lineage	
	Phagosome	

identified as hub common susceptibility genes were the targets of daclizumab and glatiramer acetate, respectively, which were approved drugs of MS. Besides, hub common susceptibility genes *JAK1* and *TYK2* were the targets of filgotinib and tofacitinib, which were under investigation for SS treatment (**Table 6**). There was no intersection between target genes of MS investigational drugs/SS-approved drugs and hub common susceptibility genes.

4 DISCUSSION

We observed the comorbidity of MS with SS or specific antibodies of SS in clinical work. Combining with studies showing their similarities in environmental factors and pathogenesis, we speculated that MS

and SS may have common genetic susceptibility factors and molecular mechanisms, which lack relevant reports. In this study, we analyzed their common genes and pathways based on GWAS data and transcriptome data and identified candidate drugs targeting common pathways and genes. In addition, a comparison between target genes of approved/investigational drugs and common susceptibility genes was performed to explore whether the drugs of MS and SS can provide reference for each other.

Through the analysis of GWAS data, 28 common susceptibility pathways of MS and SS were obtained, in which immune system-related pathways such as the "Th1, Th2 and Th17 cell differentiation" accounted for 21.43%, indicating that T cells played an important role in the pathogenesis of MS and SS. GO analysis of common susceptibility genes showed that the most significant BP was the

TABLE 4 | Drugs targeting more than or equal to two common susceptibility genes. Black-bordered letters represent drugs targeting more than or equal to two hub common susceptibility genes.
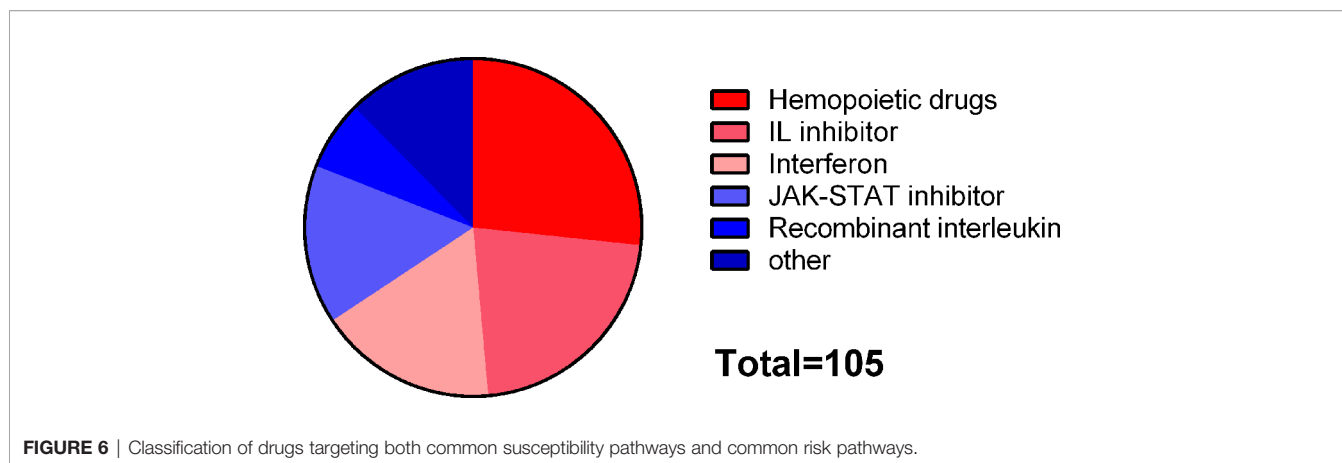
Drug	Target gene	Effect
Amoxicillin	HLA-DQB1, HLA-DRA, HLA-DRB5	Antibacterial
Aspirin	HLA-DPB1, HLA-DQB1	NSAID
Clavulanic acid	HLA-B, HLA-DQB1, HLA-DRA, HLA-DRB5	β-lactamase inhibitor
Clozapine	HLA-DPB1, HLA-DRB5	Antipsychotic
Filgotinib	JAK1, TYK2	JAK inhibitor, Anti-inflammatory
Floxacillin	HLA-B, HLA-DQB1, HLA-DRA	Antibiotic
Lumiracoxib	HLA-DQA1, HLA-DQB1	NSAID
Tofacitinib	JAK1, TYK2	JAK inhibitor
Aldesleukin	IL2RA, CD28, SOCS1, STAT4	Recombinant analog of IL-2
Arsenic trioxide	MAPK1, MAPK3	Chemotherapeutic
Baminercept	LTBR, TNFSF14	Antirheumatic
Belatacept	CD86, CD28	Selective costimulation modulator, Antirheumatic, Immunosuppressant
Briakinumab	IL12B, IL12A	Anti-IL-12 antibody
Fostamatinib	JAK1, CAMK2G, LCK	Spleen tyrosine kinase inhibitor
Leucovorin	GATA3, RUNX3	Folic acid analogs
Mercaptopurine	GATA3, HLA-DQA1	Antineoplastic
Methotrexate	SLAMF1, TNFAIP3	Antineoplastic
Purvalanol	MAPK1, MAPK3	MAPK1, MAPK3 inhibitor
Ruxolitinib	JAK1, IL7R	JAK1/2 inhibitor, Antineoplastic
Seliciclib	MAPK1, MAPK3	CDK inhibitor
Ulixertinib	MAPK1, MAPK3	ATP-competitive ERK1/2 inhibitor
Ustekinumab	IL12B, IL12A, TNFAIP3	Anti-IL12/IL23 antibody

"interferon-gamma-mediated signaling pathway", which also supported the vital contribution of T cells. Because we all know that INF-γ is the main pro-inflammatory factor of T cells. Th1 and Th17 cells were activated and enhanced in the brain tissue, cerebrospinal fluid, and peripheral blood of MS patients, of which INF-γ and interleukin-17 (IL-17) played a vital role in the pathogenesis of MS as their main cytokines (19). CD4+ T cells also made a valuable contribution to SS, especially in the early periods, as it was reported that it accounted for more than 75% of the infiltration around the salivary gland (SG) epithelial cells of SS patients (20). INF-γ that is secreted by Th1 cells upregulated the expression of *major histocompatibility complex II (MHC II)* and *CXC motif chemokine receptor 3 (CXCR3) ligands* on the surface of epithelial cells, resulting in more recruitment of pathogenic T cells to the lesions and aggravation of inflammation. Besides, highly activated Th17 cells in the peripheral blood, salivary gland, and labial gland were observed in SS patients (21). Our study found that pathways

related to infectious diseases accounted for 32.14% of the common susceptibility pathways, indicating that MS and SS both associated with infections such as EBV and human T-cell leukemia virus 1 (HTLV1), which may be the initiating factors of autoimmune responses in genetically susceptible individuals. EBV is one of the most studied viruses. Infectious mononucleosis caused by EBV infection doubled the risk of MS and increased Epstein–Barr nuclear antigen 1 (EBNA1) antibody in serum, and EBV DNA and antibodies in brain tissue were also found in MS patients (22). At the same time, the increased EBV DNA in salivary gland tissue and anti-EBV antibodies in serum were also found in SS patients. Besides, there was a relationship between the serological characteristics of previous EBV infection and anti-SSA and anti-SSB antibodies (23). Some studies showed that EBV infection of B cells was related to MS, while infection of epithelial cells was related to SS (24, 25). In addition, pathways related to autoimmune diseases also accounted for 32.14% of the common susceptibility pathways, suggesting that

TABLE 5 | Drugs targeting more than or equal to two common DEGs. Black-bordered letters represent drugs targeting more than or equal to two hub DEGs.

Drug	Target gene	Effect
Cisplatin	PIK3CA, STAT1	Antineoplastic
Doxorubicin	CYP1B1, GATA3, NT5E, PIK3CA	Antineoplastic
Vincristine	ABCB4, GATA3, PIK3CA	Antineoplastic
Benzquinamide	ABCB4, CHRM3	Antihistaminic, Anticholinergic
Curcumin	ABCB4, CAMK2A	Antibacterial, anti-inflammatory, hypoglycemic, antioxidant, wound-healing, and antimicrobial activities
Celecoxib	ALOX12, PIK3CA	NSAID
Cyclophosphamide	CYP1B1, GATA3	Antineoplastic, Immunosuppressant
Docetaxel	CYP1B1, PIK3CA	Antihistaminic
Fluorouracil	CYP1B1, PIK3CA	Antihistaminic
Letrozole	CYP1B1, PIK3CA	Aromatase inhibitor
Paclitaxel	CYP1B1, PIK3CA	Antihistaminic
Cetuximab	NT5E, PIK3CA	Antihistaminic

FIGURE 6 | Classification of drugs targeting both common susceptibility pathways and common risk pathways.

genetic and environmental factors were common factors in most autoimmune diseases.

Ten common risk pathways of MS and SS were obtained by the analysis of the transcriptome data. In particular, "JAK-STAT signaling pathway" and "influenza A" were included in both common susceptibility pathways and common risk pathways at the same time, which may participate in the pathogenesis of both MS and SS and strongly contribute to their shared mechanism. Abnormal activation of JAK-STAT signaling pathway had been observed in many autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriatic arthritis, and psoriasis. It was a pathway that mediated the signal transduction activated by a variety of cytokines, resulting in cell activation, proliferation, and the release of inflammatory factors, and also contributed primarily to the differentiation and function of Th1 and Th17 cells. Four JAKs (JAK1, JAK2, JAK3, and TYK2) and 7 STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) had been identified in mammals, which could specifically combine with each other to transduce a unique signal (26). JAK1 and STAT3 were the most significant hub common susceptibility genes in this study, TYK2 was also one of the hub common susceptibility genes, and STAT1 was one of the hub common DEGs. Enhanced activation of STAT1 and STAT3 in peripheral blood lymphocytes was observed in MS patients, indicating the activation of the pro-inflammatory response (27, 28). Moreover, highly activated STAT3 in T cells of clinically isolated syndrome (CIS) patients can predict the possibility of progression to MS (29). In addition, elevated STAT3 in brain myeloid cells was observed in MS patients and experimental autoimmune encephalomyelitis (EAE) model, proving its vital contribution to the regulation of myeloid cell function (30, 31). Moreover, selective ablation of

STAT3 made mice resistant to EAE induction by inhibiting the production of pathogenic T cells (31). Immunohistochemical analysis of labial salivary gland (LSG) in SS patients showed that the expression of JAK1 and JAK2 was enhanced in ductal cells and acinar cells, respectively. The expression of STAT1 was increased in salivary gland tissues of SS patients (32). Gene expression profile analysis of epithelial cells from SS patients' LSG also proved the significant enrichment of JAK-STAT-related genes (33), which could reduce the expression of autophagy-related gene-5 (ATG5) in LSG epithelial cells and increase the expression of pro-inflammatory factors such as IL-6 (34).

Fourteen hub common susceptibility genes were obtained by constructing pathway–gene network and PPI network. Eight of them belonged to human leukocyte antigen (HLA) class Ⅱ genes. HLA genes code for the HLA molecules that are the glycoproteins located on the surface of antigen-presenting cells and participate in immune response by recognizing and presenting exogenous (I) and endogenous (II) antigens. Variations of HLA genes contribute to genetic susceptibility of MS about 20%–60% (35). In particular, HLA-DRB1 had the strongest association with MS, which was consistent with the results of our study. Structural study showed that HLA-DRB1*1501 affected the peptide binding site of the HLA molecule and promoted combination with autoantigens like myelin basic protein, resulting in 3-fold increased risk of MS (36). The gene locus with the strongest association with SS was also located in HLA genes. However, due to racial differences, the current conclusions were not consistent. A meta-analysis integrating 23 studies showed that HLA-DRB1*03:01, DQA1*05:01, and DQB1*02:01 were all associated with the risk of SS (37). Lessard et al. (38) found that HLA-DQB1 was mostly related to primary SS in the European population, followed by HLA-DQA1. Li et al. (39) found that HLA-DRB1/HLA-DQA1 and HLA-DPB1 had the strongest association in the Chinese Han population. HLA-DRB1, HLA-DQB1, HLA-DQA1, and HLA-DPB1 were all included in the hub common susceptibility genes in our study. JAK1 and STAT3 were the most significant in non-HLA genes, which was consistent with our conclusion of pathway enrichment. In addition, Mendelian randomization study showed that there was a causal relationship between the genetic susceptibility of IL2RA and MS, whose

TABLE 6 | Approved/investigational drugs targeting hub common susceptibility genes.

Drug	Target gene	Effect
Glatiramer acetate	HLA-DRB1	Immunomodulator
Daclizumab	IL2RA	Anti-CD25(IL-2) antibody
Filgotinib	JAK1, TYK2	JAK inhibitor
Tofacitinib	JAK1, TYK2	JAK inhibitor

targeted drug daclizumab had distinct efficacy in reducing gadolinium-enhanced lesions and annual recurrence rate (40). The level of serum soluble *IL2RA* in SS patients increased significantly, which was closely related to clinical characteristics such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (41). Studies have found that microglia with overactivated *MAPK* can injure oligodendrocytes (OLs) and lead to demyelination, while *MAPK* inhibitors can promote the differentiation of OL precursor cells into mature OLs and myelination, which may become a new method for the treatment of demyelinating diseases (42). MAPK/ERK pathway is a strong stimulator for cell survival, growth, and proliferation with an important effect on anti-apoptosis, which can be affected by JAK-STAT signal pathway at the same time. Activation of MAPK/ERK pathway was also found in peripheral blood and LG cells of SS patients, and *MAPK* inhibitors could significantly reduce the production of IL-17-related pro-inflammatory factors (43). At present, polymorphism of *JAK1*, *STAT3*, *IL2RA*, *MAPK1*, and *TYK2* related to SS is not yet reported, which may become the candidate genes for SS in the future.

Through constructing a PPI network, 3 hub common DEGs were obtained, including *STAT1*, *GATA3*, and *PIK3CA*. *GATA3* is a transcription factor required for Th2 cell differentiation, which promotes the development of Th2 cells and prevents the proliferation of Th1 cells. Studies had reported that the expression of *GATA3* in peripheral blood of MS patients decreased, while the increase of its expression reduced the severity of EAE (44, 45). *PI3K* is widely expressed and participates in multiple inflammatory responses. Inhibition of *PI3K* may inhibit activated Th1/Th17 cells and microglia (46). Moreover, animal experiments showed that *PI3K* inhibitor could reduce the condition of EAE and SS (46, 47).

As for treatment, glucocorticoid is mainly used for acute attack of MS, and disease-modifying therapy (DMT) is mainly used for remission, including INF-β, glatiramer acetate, teriflunomide, dimethyl fumarate, sphingosine-1-phosphate (SP1) receptor regulator, monoclonal antibody, and chemotherapeutic drugs. In terms of treatment for SS, topical treatment is first considered, including muscarinic agonists and artificial saliva for dryness of mouth and artificial tears for dryness of eyes. In addition, glucocorticoids and cyclosporine A are considered in serious conditions. Glucocorticoids, immunosuppressants, and biological agents can be used when the system involvement appears, but they are all empirical except hydroxychloroquine.

In order to identify candidate drugs for MS or SS, we searched for the drugs targeting common pathways and genes based on GWAS data and transcriptome data, respectively. As for common pathways, we found that there were 105 overlaps between agents from GWAS and transcriptome data, of which 60.9% were immunomodulatory drugs, including IL inhibitors, JAK-STAT inhibitors, recombinant IL, and INF, suggesting their potential therapeutic effects on MS and SS, especially on their comorbidity. Besides, as for common genes, 28 overlaps between agents from GWAS and transcriptome data were obtained, among which

methotrexate, cyclophosphamide, and mercaptopurine were used for SS according to clinical experience and for MS when first-line and second-line drugs have no effect. In addition, we compared the target genes of approved/investigational drugs for MS and SS with common susceptibility genes and found that the target genes of glatiramer acetate and daclizumab were *HLA-DRB1* and *IL2RA*, respectively. Glatiramer acetate is a first-line treatment for MS, as a myelin basic protein (MBP) analog that specifically competes with myelin antigen to bind T cells. Therefore, it may have a specific immunosuppressive effect in MS. Although it can also induce Th2 cells and inhibit dendritic cells and monocytes, the immunomodulatory effect in SS needs to be further studied. Daclizumab was also used for MS but was delisted in 2018 due to its side effects of severe encephalitis and meningitis, resulting in no consideration to be used for SS. However, the possibility of *IL2RA*-targeted drugs in the treatment of MS and SS cannot be ignored.

Combining the results of identification of drugs targeting common pathways/genes and analysis of target genes of approved/investigational drugs, we suggested that JAK-STAT inhibitors may be potential common therapeutic drugs for MS and SS. At present, *JAK* inhibitors have been applied for the treatment of several autoimmune diseases, including tofacitinib, baricitinib, and upadacitinib, which have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of RA, and tofacitinib is also used for psoriatic arthritis (48). Previous studies have shown that *JAK* inhibitors play a therapeutic role in MS and SS mice. The inhibitors of *JAK1/2* such as baricitinib, ruxolitinib, and AZD148 can attenuate the severity of EAE by inhibiting Th1 and Th17 cells and inducing regulatory T (Treg) cells (49–51). Tofacitinib is a nonselective *JAK* inhibitor (*JAK1*, *JAK2*, *JAK3*, *TYK2*) and has a beneficial effect on myelination by inhibiting the inflammatory cascade (52). In a phase 2 clinical trial, the topical application of tofacitinib reduced the infiltration of inflammatory cells and expression of pro-inflammatory factors (tumor necrosis factor -α, IL-23, and IL-17A) in cornea and attenuated the dryness of eyes (53). Filgotinib is a *JAK1* inhibitor that is indicated for the treatment of active moderate to severe rheumatoid arthritis alone or in combination with methotrexate. After treatment with filgotinib, significant increase in salivary secretion and decrease in lymphocyte infiltration were observed in SS mice (54). The clinical trials of filgotinib (NCT03100942) and baricitinib (NCT04916756) for SS are underway, and the results are expecting. In addition, *STAT* inhibitors (such as Statatic) was found to attenuate the symptoms and inflammatory indicators of EAE. Therefore, JAK-STAT inhibitors may have therapeutic effects on patients with MS or SS. In particular, JAK-STAT inhibitors used for MS/SS may also contribute to the therapy of SS/MS and may also become a choice for the comorbidity of MS and SS. However, there is a case report of reversible multifocal CNS demyelination in a patient with seropositive RA during tofacitinib therapy (55). So, it is worthwhile to further study the role of JAK-STAT pathway in MS and therapeutic effect of JAK-STAT inhibitors in MS.

This study had some limitations, as follows. First, although GWAS can identify multiple SNPs related to diseases, it only can

reveal a small part of genetic factors related to complex diseases, and there existed racial heterogeneity. However, GWAS is one of the main methods to find genetic variations of complex diseases at present. Second, only two GWAS reports were available, leading to a small number of SS susceptibility genes. Therefore, when analyzing GWAS data, we first performed the pathway enrichment of disease susceptibility genes and then obtained the MS or SS susceptibility genes on the common susceptibility pathways, which were defined as the common susceptibility genes in this study. Third, the cutoff for DEGs was relatively low in our study (*P*-value <0.05 and fold change ≥1.2). When we increased the fold change to 1.3 as used for common cases and made subsequent analysis, the common DEGs of MS and SS decreased to 40 including 3 DEGs in CD4$^+$ T cells and 37 DEGs in B cells. The detailed results of fold change ≥1.3 and comparison with the results of fold change ≥1.2 were shown in **Supplementary Table S9**. GO annotation showed that the most significant BP was "positive regulation of mast cell degranulation"; the only significant CC was "cytoplasm," and the only significant MF was "double-stranded DNA binding" (**Supplementary Figure S1**). The PPI network with 11 nodes and 8 edges was constructed, and one hub common DEG (*STAT1*) with degree ≥4 was identified (**Supplementary Figure S2**). In terms of KEGG pathway enrichment, 24 and 43 significant risk pathways of MS and SS as well as 6 common risk pathways were obtained (**Supplementary Table S10**). Although the cutoff for DEGs was relatively low, the results based on transcriptome data were meaningful to a certain extent, especially combined with the results based on GWAS data.

5 CONCLUSION

Our study revealed the shared mechanism of MS and SS based on the available GWAS data and transcriptome data. Seventeen genes such as *HLA-DRB1*, *JAK1*, *STAT3*, and *STAT1* and JAK-STAT signal pathway have been identified as key genes and pathway in the shared mechanism, which may be the genetic and molecular bases of clinical comorbidity of MS with SS.

Importantly, JAK-STAT inhibitors may become potential therapeutic drugs for MS and SS, especially for their comorbidity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

XXH, XW, XMR, TTZ, and JF: conceptualization. XXH, XW, XYY, and JF: data curation. XXH, XMR, RW, TTZ, and JF: formal analysis. XXH, XMZ, RW, and JF: funding acquisition. XXH: writing—original draft. XXH, DW, TTZ, and JF: writing—review and editing. All authors read and approved the final article.

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Supplementary Figure S1 | GO terms in the biological process, cellular component, and molecular function category of common DEGs (Fold change ≥ 1.3).

Supplementary Figure S2 | Protein-protein interaction network of common DEGs (Fold change ≥ 1.3). Nodes represent DEGs, edges represent combined score of PPI. The higher the combined score, the thicker the edge.

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Perspectives of JAK Inhibitors for Large Vessel Vasculitis

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Vasculitis is an inflammation of the blood vessels caused by autoimmunity and/or autoinflammation, and recent advances in research have led to a better understanding of its pathogenesis. Glucocorticoids and cyclophosphamide have long been the standard of care. However, B-cell depletion therapy with rituximab has become available for treating antineutrophil cytoplasmic antibody-associated vasculitis (AAV). More recently, avacopan, an inhibitor of the complement 5a receptor, was shown to have high efficacy in remission induction against AAV. Thus, treatment options for AAV have been expanded. In contrast, in large vessel vasculitis (LVV), including giant cell arteritis and Takayasu arteritis, tocilizumab, an IL-6 receptor antagonist, was shown to be effective in suppressing relapse and has steroid-sparing effects. However, the relapse rate remains high, and other therapeutic options have long been awaited. In the last decade, Janus kinase (JAK) inhibitors have emerged as therapeutic options for rheumatoid arthritis (RA). Their efficacy has been proven in multiple studies; thus, JAK inhibitors are expected to be promising agents for treating other rheumatic diseases, including LVV. This mini-review briefly introduces the mechanism of action of JAK inhibitors and their efficacy in patients with RA. Then, the pathophysiology of LVV is updated, and a rationale for treating LVV with JAK inhibitors is provided with a brief introduction of our preliminary results using a mouse model. Finally, we discuss the newly raised safety concerns regarding JAK inhibitors and future perspectives for treating LVV.

Keywords: giant cell arteritis, JAK inhibitors, large vessel vasculitis, Janus kinase (JAK), Takayasu arteritis

INTRODUCTION

Vasculitis syndrome is an autoimmune and/or autoinflammatory condition that causes inflammation of the blood vessels, and the resultant tissue ischemia causes damage to various organs. It is classified as large-, medium-, and small-vessel vasculitis according to the size of the affected blood vessels. The mainstay of treatment for vasculitis has long been glucocorticoids (GCs) and immunosuppressive agents such as cyclophosphamide, azathioprine, and methotrexate (MTX). However, new treatment options have long been awaited because of the significant burden of side effects of the treatment.

Treatment options for antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, particularly in microscopic polyangiitis and granulomatosis with polyangiitis, have expanded considerably in recent years. For example, B-cell depletion therapy using rituximab is as effective and safe as cyclophosphamide (1, 2). More recently, the efficacy and safety of avacopan, a

complement 5a (C5a) receptor inhibitor that blocks neutrophil chemoattraction and activation, has been examined in ANCA-related vasculitis (3). This study showed that the C5a receptor blockade was superior to standard steroid therapy in remission induction at week 52, suggesting that avacopan may have the potential to replace standard steroid therapy (4).

In contrast, in large vessel vasculitis (LVV), including giant cell arteritis (GCA) and Takayasu arteritis (TAK), tocilizumab (TCZ), an IL-6 receptor antibody, is effective in preventing recurrence and reducing the dose of GCs (5, 6). However, the primary endpoint was not met in TAK (5), and many patients experienced relapse after discontinuation of TCZ (7), necessitating treatment that fundamentally improves vascular inflammation. Moreover, blockade of T cell costimulation signals using abatacept is effective and safe in GCA (8), but failed to show its efficacy in TAK (9). TAK often affects young women, and the side effects of accumulated steroids owing to multiple relapses are immense (10). Many of the drugs used in real-world clinical practice for TAK lack sufficient evidence in randomized controlled trials (11). Thus, unmet clinical needs remain for LVV, particularly in TAK.

In the last 10 years, Janus kinase (JAK) inhibitors have emerged as promising agents for rheumatology (12). JAK inhibitors are low-molecular-weight compounds that can be orally administered to patients with rheumatoid arthritis (RA), unlike biological disease-modifying antirheumatic drugs (bDMARDs) (13). Their efficacy and safety have been compared with those of bDMARDs and have been proven in multiple studies in patients with RA.

This mini-review briefly explains the mechanism of action of JAK inhibitors and their efficacy in patients with RA. Then, we update the pathophysiology and provide a rationale for treating LVV with JAK inhibitors. Finally, we discuss the safety and future perspectives of JAK inhibitors for LVV treatment.

SUCCESS OF JAK INHIBITORS IN RA

Mechanism of Action

Cytokine receptors are grouped into several superfamilies based on their shared structural elements of the receptors (14). Type I and type II cytokines utilize the JAK-signal transducer and activation of transcription (STAT) pathway (**Figure 1**). When type I and II cytokines bind to their receptors on the cell surface, JAKs bound to the intracellular domains are phosphorylated by adenosine triphosphate binding, which in turn phosphorylates the receptor end. The transcription factor STAT binds to the receptor end, and phosphorylated STATs form a dimer, which is
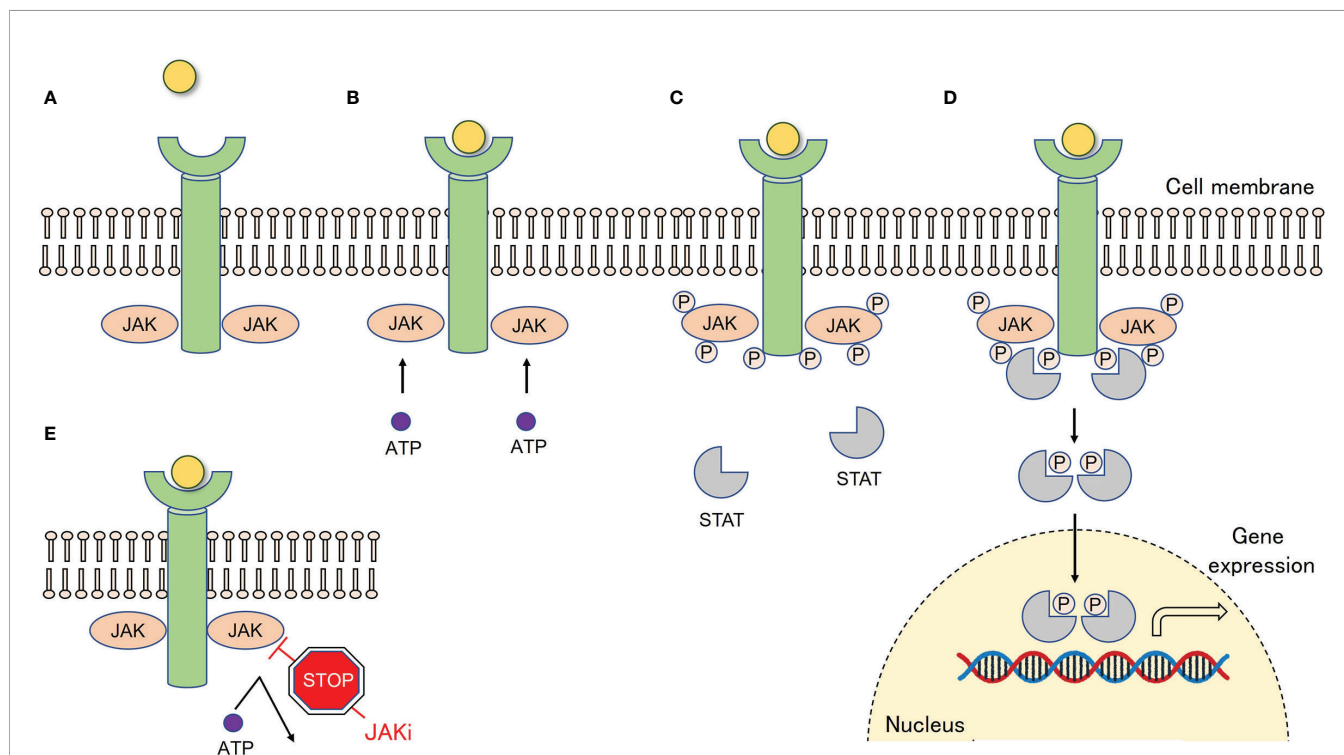
FIGURE 1 | The JAK-STAT pathway and mechanism of action of JAK inhibitors. **(A)** Type I and type II cytokines utilize the Janus kinase (JAK)-signal transducer and activation of transcription (STAT) pathway. **(B)** When type I and II cytokines bind to their receptors on the cell surface, JAKs bound to the intracellular domains are phosphorylated by adenosine triphosphate binding. **(C)** Phosphorylated JAKs, in turn, phosphorylate the receptor end. **(D)** The transcription factor STAT binds to the receptor end, and phosphorylated STATs form a dimer, which is then transferred to the nucleus to regulate gene expression. **(E)** JAK inhibitors competitively bind to the binding site of ATP, inhibiting phosphorylation of JAK and exerting their effects. ATP, Adenosine triphosphate; JAK, Janus kinase; JAKi, Janus kinase inhibitor; P, Phosphate; STAT, Signal transducer and activator of transcription.

then transferred to the nucleus to regulate gene expression (15). There are four isoforms of JAKs (JAK1, JAK2, JAK3, and TYK2). Type I and type II cytokines include the common γ chain family (IL-2, 4, 7, 9, 13, and 15), gp130 cytokines (IL-6, Oncostatin M), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, β, γ, IL-12, and others, but not tumor necrosis factor α (TNF-α), IL-1, IL-17, and TGF-β (12).

Efficacy of JAK Inhibitors in RA

The efficacy of JAK inhibitors has been tested in head-to-head comparisons with adalimumab, a representative TNF-α inhibitor, in multiple trials involving patients with RA. The results demonstrated that JAK inhibitors are non-inferior or even superior to adalimumab in controlling disease activity (16–18). Based on these results, JAK inhibitors have been placed equal to bDMARDs in the most updated RA treatment recommendations (19). In other words, when methotrexate fails to induce remission, RA patients can choose either bDMARDs or JAK inhibitors. Thus, JAK inhibitors are an essential therapeutic option for the treatment of RA.

JAK INHIBITORS FOR VASCULITIS

Large Vessel Vasculitis: GCA and TAK

Both GCA and TAK affect the aorta and its major branches and are characterized by granulomatous vascular inflammation (20). IFN-γ and IL-17 derived from Th1 and Th17 cells are the dominant cytokines (21–23), and neoangiogenesis or new formation of vasa vasorum in the adventitia and lumen occlusion due to intimal hyperplasia can be observed in both diseases (24). Although many disease mechanisms are shared, several differences exist. For example, granulomatous lesions mainly contain CD4^{+} T cells and macrophages in GCA, whereas CD8^{+} T and NK cells are also involved in TAK (25). In the peripheral blood, the follicular helper T cell-B cell signature, which promotes immunoglobulin production, is highly enriched in TAK, but not in GCA (26). Adventitial fibrosis is more prominent in TAK than in GCA (25). Thus, from a pathomechanistic point of view, TAK is more complex than GCA and a single therapeutic target alone may not be sufficient to achieve remission. In this context, JAK inhibitors are expected to be effective because of the simultaneous blockade of multiple cytokines, especially in TAK.

Our previous work showed enhanced activity of the JAK-STAT pathway in the vascular lesions of patients with GCA (27). Compared with biopsy-negative temporal arteries, biopsy-positive temporal arteries showed elevated transcripts of STAT1, STAT2, and STAT4, as well as target genes corresponding to each STAT. Moreover, cytokine production in CD4^{+} T cells from patients with GCA was dependent on the JAK-STAT pathway, as demonstrated by an experiment showing that tofacitinib, an inhibitor of JAK1 and JAK3, inhibited IFN-γ production in a dose-dependent manner (27). In line with this report, a recent study from a French group demonstrated that

STAT1 and STAT2 transcripts were highly upregulated in aortic lesions of GCA by using microarray analysis (28). In addition, both CD4^{+} and CD8^{+} T cells in the peripheral blood of patients with GCA showed increased activity of the JAK-STAT pathway. The same group also identified upregulated JAK-STAT signals in both CD4^{+} and CD8^{+} T cells in the peripheral blood of patients with TAK (29).

What is the mechanism underlying the enhanced activity of the JAK-STAT pathway in LVV (**Figure 2**)? This question is equivalent to asking which type I and II cytokines are implicated in GCA and TAK. Undoubtedly, IL-6 plays a key role in both diseases, as suggested by the clinical effects of TCZ (5, 6). IL-6 is mainly derived from CD68^{+} tissue macrophages in both GCA and TAK (30, 31), and IL-6 primarily utilizes STAT3 as a downstream transcription factor (32). Since the above-mentioned studies have demonstrated that STAT3 is highly activated in CD4^{+} and CD8^{+} T cells in both diseases (28, 29), this IL-6-STAT3 axis substantially contributes to the pathogenesis of both diseases. However, this axis alone does not explain the increased activity of STAT1 and STAT2 signals in the vascular lesions of the GCA (27, 28).

In recent years, type I IFNs have attracted attention in the pathophysiology of LVV. Upregulation of type I IFNs, particularly IFN-α, has been reported in the serum, temporal arteries, and aortic lesions of GCA (27, 28). A highly enriched type I IFN signature has been observed in both CD4^{+} and CD8^{+} T cells from TAK patients (29). The binding of type I IFNs to their receptors activates JAK1 and TYK2, which is followed by the phosphorylation of STAT1 and STAT2 (33), which fits perfectly into the context of what has been reported so far. Although plasmacytoid dendritic cells are the main source of type I IFNs in systemic lupus erythematosus, those in GCA and TAK remain unknown (34). In addition to type I IFNs, the role of GM-CSF in the promotion of vascular inflammation in GCA has been reported (35). Thus, IL-6, type I IFNs, and GM-CSF are involved in the pathogenesis, all of which utilize the JAK-STAT pathway, making it highly likely that JAK inhibitors are effective against GCA.

Furthermore, a genome-wide association study identified IL-12B as a susceptibility gene for TAK (36, 37). Serum IL-12 levels are elevated in TAK patients (38), and the risk allele of IL-12B is closely associated with vascular damage in TAK (39). IL-12 uses the JAK-STAT pathway, and JAK2 and TYK2 are located in the downstream signaling pathway (40). Thus, JAK inhibitors are expected to be effective against TAK (41).

Based on these findings, we examined the effects of tofacitinib, which blocks JAK1 and JAK3, on LVV in a mouse model (27). In this mouse model, human medium-sized arteries were engrafted into immunodeficient mice, and vascular inflammation was induced by injecting lipopolysaccharide and peripheral blood mononuclear cells from patients with GCA. In this model, tofacitinib not only inhibited T-cell activation and cytokine production but also inhibited macrophage activation, resulting in the efficient suppression of vascular inflammation. Analysis of T cells in vasculitic lesions identified a highly proliferative population, called "tissue-resident memory T (Trm) cells".
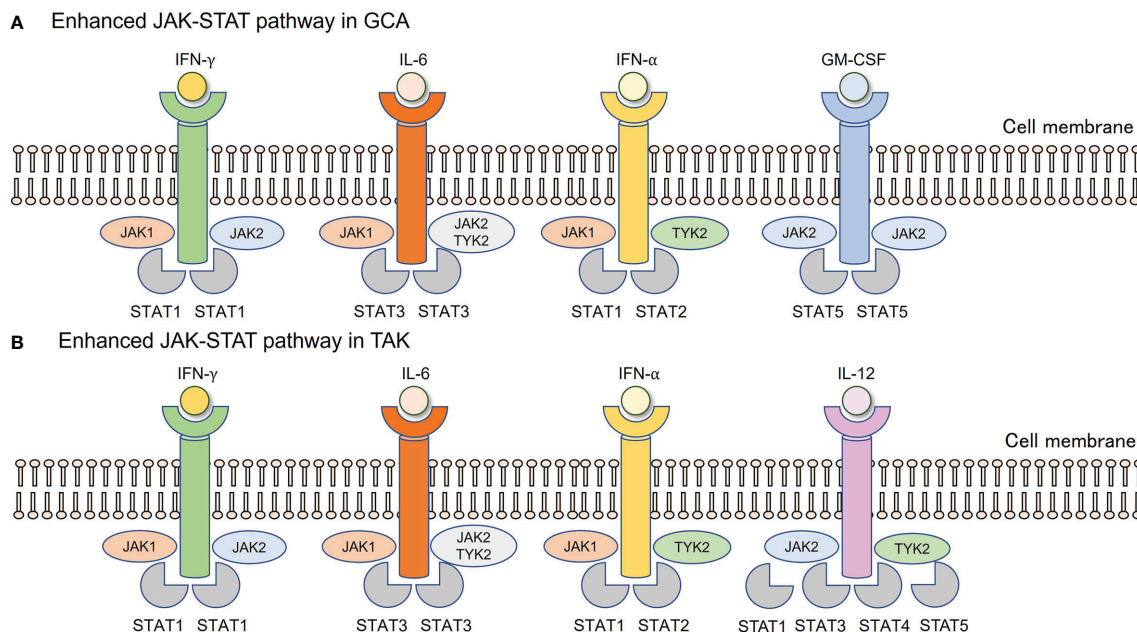
FIGURE 2 | Enhanced JAK-STAT pathway in GCA and TAK. **(A)** In vascular lesions of GCA, IFN-γ derived from Th1 cells, IL-6 from tissue macrophages, IFN-α, and GM-CSF derived from unknown origin are enriched. **(B)** In vascular lesions of TAK, IFN-γ derived from Th1 cells, IL-6 from tissue macrophages, IFN-α from unknown origin, and IL-12 from monocyte/macrophages are enriched. Each cytokine utilizes its JAKs, followed by corresponding STAT phosphorylation. GCA, Giant cell arteritis; GM-CSF, Granulocyte macrophage colony-stimulating factor; IFN, Interferon; JAK, Janus kinase; STAT, Signal transducer and activation of transcription; TAK, Takayasu arteritis.

Trm cells express CD69 and CD103 and show a rapid response to antigens once encountered. These cells may have the potential to induce a relapse of vascular inflammation in GCA (42). Our results demonstrate that these cells can be targeted by tofacitinib as well (27).

In line with these data from basic research, several case reports describing the efficacy of JAK inhibitors on LVV have been published (29, 43–48). Very recently, baricitinib, an inhibitor of JAK1 and JAK2, was reported to be effective against relapsing GCA in a prospective open-label study (49). Although the number of enrolled patients was small, the high remission induction and steroid withdrawal rates suggest that this treatment is promising for GCA. In addition, the efficacy and safety of tofacitinib and MTX were prospectively evaluated in active Takayasu arteritis (50). Compared to MTX-treated group, complete remission and steroid reduction rates were higher in the tofacitinib-treated group, but relapse and imaging improvement rates did not reach the statistical significance. Other clinical trials of JAK inhibitors for GCA (NCT03725202, upadacitinib) and TAK (NCT04161898, upadacitinib) are ongoing. TAK may be less likely to produce good results than GCA because of the complexity of the disease mechanism; however, we are awaiting promising results.

Other Forms of Vasculitis

Once the efficacy of JAK inhibitors has been experimentally demonstrated in LVV, they are expected to be effective in other forms of vasculitis. Some pilot studies and case reports demonstrated the efficacy of JAK inhibitors for ANCA-associated vasculitis (51), polyarteritis nodosa (52), cutaneous leukocytoclastic vasculitis (53), and vascular Behcet's disease (54); however, data on other forms of vasculitis are very limited (55), and we cannot get any conclusion from such limited data.

DISCUSSION

So far, we have focused on the efficacy of JAK inhibitors for rheumatic diseases. As for safety, data are accumulating on the treatment of RA. The use of JAK inhibitors is associated with a higher risk of developing shingles, reactivation of varicella-zoster virus (VZV), than bDMARDs (56). An increased risk of serious infections compared to bDMARDs has also been reported in some trials (57). In addition, new safety concerns emerged after the results of an Oral Surveillance trial were published (58). In this trial, patients with active RA who were at risk for cardiovascular events, such as smoking, were assigned to one of three treatment groups: TNF inhibitors, or 5 mg of tofacitinib twice daily, or 10 mg twice daily, and observed for 5 years. The results showed an increased risk of death, malignancy, major adverse cardiac events (MACE), and venous thromboembolism (VTE) in tofacitinib-treated patients (both 5mg and 10 mg arms) compared to those treated with TNF inhibitors (58). In September 2021, the Food and Drug Administration issued a warning regarding the use of JAK inhibitors. Subsequently, the

use of JAK inhibitors for patients with RA is, in principle, limited to patients who are refractory to at least one TNF inhibitor. Although selection bias, which only recruited patients at risk of cardiovascular events, cannot be ruled out in the study, and real-word data from a large cohort do not support the increased risk of such serious adverse events (59), we agree that screening before administration and regular monitoring during administration are essential for the treatment with JAK inhibitors.

GCA patients are often older than RA patients and are at higher risk of serious infection, MACE, and VTE (60–62). Therefore, it is recommended that JAK inhibitors be administered only after adequate risk management and cardiovascular prevention. With regard to shingles, it has been reported that VZV is a contributing factor in the development of GCA (63, 64) and is considered extremely high-risk in elderly patients with GCA. In the study of baricitinib for GCA described above, it was reported that the live-attenuated zoster vaccine did not prevent the onset of shingles (49). It has been reported that recombinant adjuvanted zoster vaccine can suppress the onset of herpes zoster at a high rate in RA patients (65). Therefore, administration of this recombinant vaccine prior to the use of JAK inhibitors is desirable in patients with GCA.

In conclusion, the efficacy of JAK inhibitors in treating rheumatic diseases is promising. Given their pathophysiology, JAK inhibitors should have high efficacy for GCA and TAK.

Therefore, the results of these clinical trials are awaited. However, new safety concerns have emerged that may be limited to treatment-resistant cases. There is an urgent need to establish the long-term safety of JAK inhibitors.

AUTHOR CONTRIBUTIONS

RW drafted the manuscript. MH revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Tofacitinib Downregulates TNF and Poly(I:C)-Dependent MHC-II Expression in the Colonic Epithelium

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Major Histocompatibility Complex (MHC)-I and -II genes are upregulated in intestinal epithelial cells (IECs) during active inflammatory bowel diseases (IBD), but little is known about how IBD-relevant pro-inflammatory signals and IBD drugs can regulate their expression. We have previously shown that the synthetic analog of double-stranded RNA (dsRNA) Polyinosinic:polycytidylic acid (Poly(I:C)), induces interferon stimulated genes (ISGs) in colon organoids (colonoids). These ISGs may be involved in the induction of antigen presentation. In the present study, we applied colonoids derived from non-IBD controls and ulcerative colitis patients to identify induction and effects of IBD-drugs on antigen presentation in IECs in the context of Tumor Necrosis Factor (TNF)-driven inflammation. By RNA sequencing, we show that a combination of TNF and Poly (I:C) strongly induced antigen-presentation gene signatures in colonoids, including expression of MHC-II genes. MHC-I and -II protein expression was confirmed by immunoblotting and immunofluorescence. TNF+Poly(I:C)-dependent upregulation of MHC-II expression was associated with increased expression of Janus Kinases *JAK1/2* as well as increased activation of transcription factor Signal transducer and activator of transcription 1 (*STAT1*). Accordingly, pre-treatment of colonoids with IBD-approved pan-Janus Kinase (JAK) inhibitor Tofacitinib led to the downregulation of TNF+Poly(I:C)-dependent MHC-II expression associated with the abrogation of STAT1 activation. Pre-treatment with corticosteroid Budesonide, commonly used in IBD, did not alter MHC-II expression. Collectively, our results identify a regulatory role for IBD-relevant pro-inflammatory signals on MHC-II expression that is influenced by Tofacitinib.

Keywords: intestinal epithelium, organoids, tumor necrosis factor (TNF), polyinosinic:polycytidylic acid Poly(I:C), antigen presentation, major histocompatibility class II, tofacitinib, ulcerative colitis

INTRODUCTION

Inflammatory bowel diseases (IBD) encompassing Crohn's disease (CD) and ulcerative colitis (UC) are associated with chronic relapsing intestinal inflammation. The pathobiology of IBD is incompletely understood but involves a disturbed crosstalk between intestinal epithelium, microbiota and host immune system in a genetically susceptible host (1, 2). Much of the focus in the past has been drawn to investigating the involvement of immune cells in IBD pathobiology. More recently, the intestinal epithelial cells (IECs) are emerging as crucial immune modulators and identified as an essential target in IBD treatment (3).

As immune modulators, IECs are non-professional antigen-presenting cells that present self and antigenic peptides to the host immune cells *via* the expression of Major Histocompatibility Class (MHC) I and II complexes. Three different isoforms of MHC-II exist in humans: HLA-DP, HLA-DR and HLA-DQ, encoded by α and β chains and their expression is highly controlled by MHC-II transactivator CIITA (4, 5). Once synthesized, the MHC-II molecules assemble in the endoplasmic reticulum upon association with invariant chain CD74 (6). This complex further translocates to the acidic endosomal compartment where the invariant chain is trimmed to class II invariant chain-associated peptide (CLIP) with the help of lysosomal protease cathepsin S (CTSS) (7, 8). Subsequently, the non-classical HLA-DM protein associates with CLIP to catalyze the exchange of CLIP with antigenic peptides in the peptide-binding groove of MHC-II that translocate to the cell surface for antigen presentation to the adaptive T cells (9–11).

MHC-II expression by IECs is critical for regulating CD4+ T cells in the lamina propria (12) and differentiation of intraepithelial lymphocytes (13, 14) that are found between the IECs. We (15) and others (16–18) have documented that MHC-II expression is upregulated in the colonic epithelium during active IBD. MHC-II expression in IECs is induced predominantly by Interferon gamma (IFNγ) from immune cells invading the mucosa during active inflammation in IBD (19–22). IFNγ signals *via* the Janus Kinases 1/2, Signal transducer and activator of transcription 1, and Interferon regulatory factor 1 (JAK1/2-STAT1-IRF1) pathway to upregulate MHC-II expression (23). Studies in murine models have revealed that IEC-specific MHC-II expression requires the presence of microbiota and microbial-driven Toll-like receptor signaling proteins TRIF and MYD88 (24, 25). These signals may further induce IFNγ protein expression from immune cells to upregulate MHC-II expression in IECs.

IBD is associated with enhanced Tumor necrosis factor (TNF) signaling and excessive epithelial cell death. It is unknown whether RNA signals derived from dead cells, such as damage-associated molecular patterns (DAMP), can impact antigen presentation in IECs. Additionally, whether viral infections in IBD can impact MHC-II expression is uninvestigated, particularly in the context of TNF (26). In a transcriptomic time-series screen using colonic HT-29 cells treated with TLR 1-9 ligands, NOD2 ligand, or cytokines (IL1β, IL10, TNFα), we found that TNF and TLR3 ligand Poly (I:C) that mimics viral and DAMP signals could upregulate MHC-I genes and MHC-II associated invariant chain CD74 (27). In addition, colon organoids (colonoids) stimulated with TNF and Poly(I:C) induced signature type I Interferon (IFN) Stimulated Genes (ISGs) (28). These observations suggest that pro-inflammatory signals such as from TNF or dsRNA may directly drive the expression of genes relevant to antigen presentation in colonic epithelium (15, 27).

IBD is associated with overexpression of different inflammatory cytokines, and several cytokines may be regulated *via* the JAK-STAT signaling pathway (29). Recently, small-molecule JAK inhibitors that inhibit phosphorylation and activation of STAT proteins have emerged as a promising therapy, particularly in UC patients (30–34). Currently, Tofacitinib, an oral pan-JAK inhibitor that is approved for use in UC refractory to standard treatment, has been shown to alleviate symptoms in UC patients with moderate to severe disease (31, 32, 34, 35). *Ex vivo*, Tofacitinib has been shown to intercept T-cell activation by downregulating costimulatory CD80/CD86 expression downstream to IFN-type I stimulation and LPS stimulation of dendritic cells (36). Studies in human colonoids indicates that Tofacitinib also plays a central role in the prevention of IFNγ-induced barrier defects (37). Although Tofacitinib has been shown to regulate several critical pathways of IFN signaling, the role of Tofacitinib on IEC-dependent antigen presentation has not been evaluated.

MHC-II expression in the context of IBD-associated intestinal inflammation, particularly in human colonic epithelium, remains vastly under-investigated. The roles of antigen presentation by IECs have mostly been studied in epithelial cancer cell lines which do not carry the same cellular composition as *in vivo* IECs, or in animal models (12, 14, 24, 25, 38) that do not fully reflect IBD pathobiology (39). A promising model for addressing human IBD-pathobiology and IBD-drug response in intestinal epithelium is intestinal epithelial organoids that recapitulate *in vivo* IECs-specific functional characteristics (28, 40–43). Recently, Wosen et al. (19), showed that intracellular MHC-II peptide-pathway is intact and functional in organoids from small intestine (enteroids). Here we evaluate how the expression of MHC-II genes is regulated by IBD-relevant pro-inflammatory signal TNF and Poly(I:C) in colonoids, and how they are modulated in a donor-specific manner by JAK inhibition using the IBD-approved drug Tofacitinib.

METHODS

Materials are listed in **Supplementary File SF1**, sheet 2.

Ethical Considerations

The current study was carried out under relevant approvals by the Central Norway Regional Committee for Medical and Health Research Ethics (reference numbers 5.2007.910 and 2013/212/REKmidt). All patients included in the study provided informed written consent.

Patient Material

Patients were admitted to the Department of Gastroenterology and Hepatology at St Olav's University Hospital, Trondheim, Norway, for colonoscopy and clinical assessment, and were diagnosed with UC or categorized as non-IBD controls. Pinch biopsies from patients were collected in 10% buffered formalin and histopathology was evaluated as described before (44). Colonoids for functional assays were established using pinch biopsies collected from uninflamed mucosa at the hepatic flexure (28, 43). Patient characteristics are listed in **Table 1**.

Colonoid Culture, Stimulation Experiments and Drug Treatment

Colonoid cultures were established and grown in 20% O_2 and 5% CO_2 at 37°C as described in Skovdahl et al. (43), based on protocols of Mahe et al. (40), and Jung et al. (45). Briefly, epithelial crypts isolated from biopsies were resuspended in ice-cold basement membrane matrix Matrigel GFR (Corning®, #734–1101, Corning, NY, USA) and 50 µL per well was added onto pre-warmed 24-well plates. Complete growth media (CGM) for the colonoids was composed of 50% Wnt-3A conditioned medium (Wnt 3A-CM, #CRL-2647, ATCC, Manassas, VA, USA), 30% Advanced DMEM/F12 (#12634028, Thermo Fischer Scientific, Bremen, Germany), and 20% R-spondin conditioned medium (HA-R-Spondin1-Fc 293T Cells, #AMS.RSPO1-CELLS, AMS Biotechnology, Abington, UK) with 10% bovine serum albumin (BSA), supplemented with N-Acetyl-L-cystein (163.2 µg/mL, #A9165-25G, Sigma-Aldrich, MO, USA), Nicotinamide (1221.2 µg/mL, #N3376-100G, MerckMillipore, Burlington, MA, USA), Recombinant Human Noggin (0.1 µg/mL, #120-10c, PeproTech, Rocky Hill, NJ, USA), TGFβ type 1 activating receptor-like kinase inhibitor (ALK5) A-83-01 (0.211 µg/mL, #SML0788, Sigma-Aldrich), MAPK inhibitor SB202190 (3.31 µg/mL, #S7067, Sigma-Aldrich), Human EGF (0.05 µg/mL, #AF-100-15, PeproTech) and Gastrin (0.02µg/mL, G9145-1MG, Sigma-Aldrich). For colonoid establishment, CGM was supplemented with Glycogen synthase kinase inhibitor CHIRR99021 (1.16 ug/mL, #72052, STEMCELL Technologies, Vancouver, Canada) and Rho kinase (ROCK) inhibitor Thiazovivin (0.78 µg/mL, #72252, STEMCELL Technologies). Colonoids were passaged every 7-8 days at least five times after establishment before carrying out the assays. General experimental outline is shown in **Figure 1A**. For all experiments, colonoids were passaged into single cells and 10,000 cells per 50 µL Matrigel was used. Colonoids were grown in CGM for ten days with selective ROCK-inhibitor Y-27632 (3.203 µg/mL, #1254, Bio-Techne, Minneapolis, MN, USA) supplemented on day one and day three. Differentiation of colonoids was initiated on day ten by lowering Wnt 3A-CM concentration to 5%, withdrawing Nicotinamide and SB202190 factor from the CGM and adding the pan-Notch inhibitor DAPT (4.324 µg/mL, #2634, Bio-Techne) with media change on alternative days. On day 14, A-83-01 was removed from the differentiation media. Tofacitinib Citrate (CP-690550) (25.2 µg/mL, #S5001, Selleckchem.com) or Budesonide (4.3 µg/mL, # S1286, Selleckchem.com) were added to colonoids in differentiation media on days 11, 13, and 14 (**Figure 1A**). Tofacitinib and Budesonide were dissolved in DMSO and 0.033%

TABLE 1 | Colonoid donor characteristics.

Donor (D)	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Diagnosis	Non-IBD	Non-IBD	Non-IBD	Non-IBD	Non-IBD	Non-IBD	UC	UC	UC	UC
Age	21	73	55	28	65	43	20	18	43	29
Sex	F	M	F	F	F	F	M	M	M	F
Medication										
5ASA	0	0	0	0	0	0	1	1	1	1
Steroid	0	0	0	0	0	0	0	1	0	1
RNA-seq Figure 1	x	x	x				x			
RNA-seq Figure 2 and Supplementary Figure 1	x	x	x				x	x	x	
Immunofluorescence Figure 3 and Supplementary Figure 2		x	x					x	x	
Immunohistochemistry Figure 3		x								
Drug treatment Figure 4 and 5							x	x		
In vivo colonic biopsy for staining*	Hepatic flexure	NA	Hepatic flexure	Hepatic flexure	Hepatic flexure	Hepatic flexure	Cecum	Ascending colon	NA	NA
Conclusion: MHC-II expression in colonic epithelium	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes

*In vivo colonic biopsies for immunostaining from the UC patiens are taken from the inflamed area. All samples for colonoids are taken from Hepatic flexure. IBD, Inflammatory bowel disease; M, Male; F, Female; UC, Ulcerative colitis; 5ASA, 5-aminosalicylic acid; RNA-seq, RNA sequencing; NA, Not available.
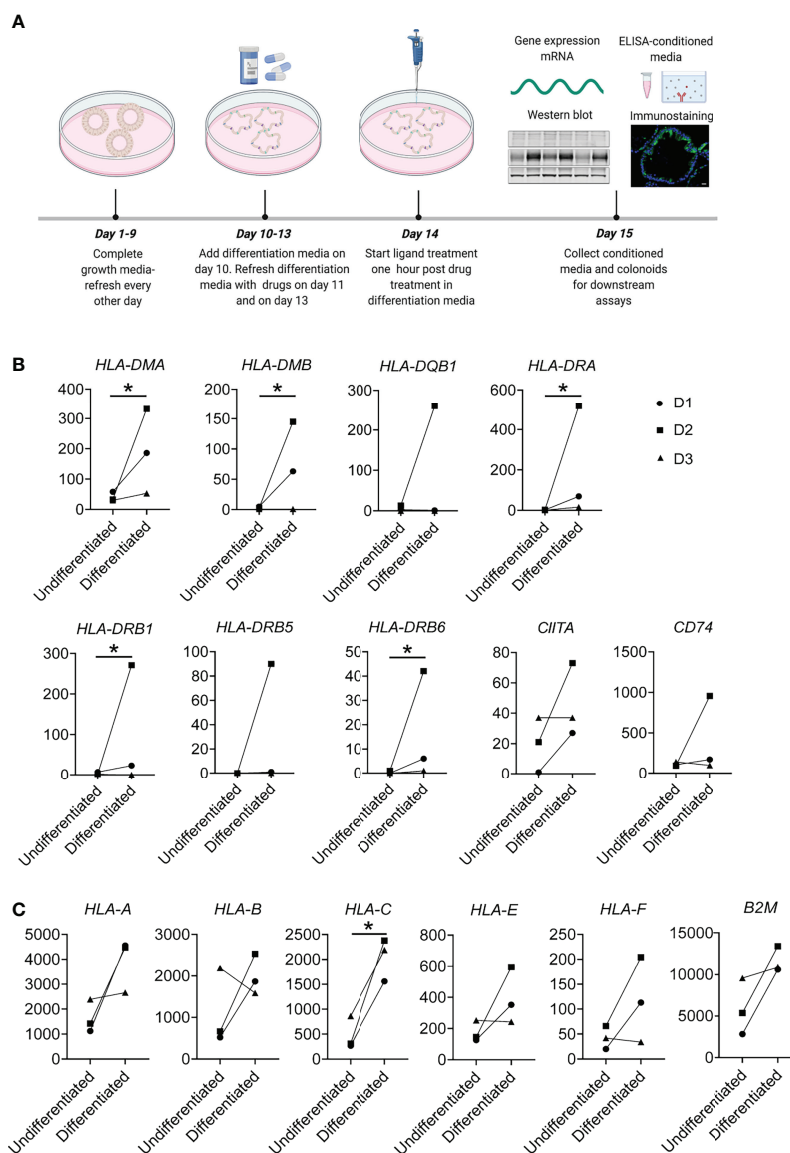
FIGURE 1 | Experimental overview and MHC-II and MHC-I expression in constitutive conditions. **(A)** General graphical representation of the experiments. Colonoids were passaged and dissociated into single cells before plating for experiments. Colonoids were given complete growth media every other day where ROCK inhibitor Y-27632 was added for the first two changes, as described in the Methods section. On Day 10, colonoids received differentiation media. For experiments involving drug treatments, 50 μM Tofacitinib, 10 μM Budesonide or DMSO (vehicle controls) were added in differentiation media on day 11 and refreshed on day 13 and one hour before stimulation with ligands TNF or TNF+Poly(I:C) on day 14. On day 15, colonoids were collected for downstream experiments, and conditioned media from colonoids for analyzing protein secretion. The illustration was created with Biorender.com. **(B)** MHC-II and **(C)** MHC-I associated genes showing increased expression in differentiated colonoids compared to undifferentiated colonoids. In this experiment, colonoids from 3 non-IBD controls were passaged and plated as single cells that were cultured in growth media for 10 days and either remained in growth media for 5 additional days (undifferentiated colonoids) or differentiated with differentiation media by reducing the WNT concentration of the media. On day 15, undifferentiated and differentiated colonoids were collected and analysed for gene expression by RNAseq. The plots show paired changes in normalized mRNA reads (Y-axis) for the different donors (D1-D3, **Table 1**). P values were obtained by LIMMA linear models with least squares regression and empirical Bayes moderated t-statistics with Benjamini Hochberg FDR correction for multiple comparisons and * indicates $P < 0.05$.

DMSO was used as vehicle control. TNF (100 ng/mL, #300-01A, PeproTech) and Poly(I:C) (20 μg/mL, #tlrl-pic, *In vivo*Gen) or IFN-γ (10 ng/mL, # 300-02, PeproTech) were added to the differentiation media and 24-hours later the conditioned media and colonoids were collected for downstream assays. Matrigel was dissolved using cell recovery solution (#734-0107, Corning, NY, United States) and colonoids were washed thrice in ice-cold Phosphate buffered saline (PBS) for immunoblotting or PBS with 0.1% BSA for RNA isolation and stored at -80°C. Alternatively, colonoids were prepared for immunostaining as described below.

RNA Sequencing and Bioinformatics

To extract RNA from colonoids, the RNeasy mini kit (#74106, Qiagen, Hilden, Germany) was used as per the manufacturer's instructions. RNA sequencing (RNA-seq) of colonoids was performed as described before (43). RNA-seq libraries were generated using SENSE total RNASeq library prep kit with RiboCop rRNA depletion (Lexogen GmbH, Vienna, Austria). Sequencing consisting of 75 single-end reads was performed using Illumina HiSeq4000 instrument (Illumina, Inc., San Diego, CA, USA), and FASTQ files were generated using bcl2fastq 2.18 (Illumina) as per manufacturer's protocols.

All RNA-seq data analysis was performed in R software. LIMMA linear models with least squares regression and empirical Bayes moderated t statistics were used to identify differential gene expression between experimental groups, and Benjamini–Hochberg false discovery rate (FDR) correction-adjusted P values ≤ 0.05 were considered statistically significant. Enrichment analyses were generated with MetaCore+MetaDrug^{TM} version 21.3 build 7060. Normalized reads and log2 values for all experiments are mentioned in **Supplementary File SF2**. The RNA-seq dataset for TNF and unstimulated conditions is available under the GEO accession number GSE172404.

Immunostaining of Biopsies and Colonoids

Patient colonic biopsies were fixed in 10% buffered formalin for 3-6 days as described previously (44). Colonoids collected in 50 µL Richard-Allan Scientific^{TM} HistoGel^{TM} Specimen Processing Gel (#HG-4000-012, Thermo Fisher Scientific, Massachusetts, USA) were fixed for 24-48 hours in 10% buffered formalin as described previously (28, 43). Formalin-fixed paraffin-embedded sections from biopsies or colonoids were treated with Neo-Clear (#1.09843.5000, MerckMillipore) and a series of ethanol for de-paraffinization. For immunohistochemistry (IHC), endogenous peroxidase was quenched with hydrogen peroxide and washed once with deionized water. Antigen retrieval was performed by boiling with citrate buffer (pH 6.0) for 15 minutes in a microwave oven. All samples were blocked with 3% BSA in 0.25% Triton-X in PBS (PBST) for 30 minutes at room temperature, washed twice in PBST, incubated overnight at 4°C with pan MHC-II-Mouse monoclonal Anti-HLA DR + DP + DQ antibody [CR3/43] (1:400 dilution in 1% BSA in PBST, #ab7856, Abcam, MA, USA) in a hydrated chamber. The following day, for secondary staining in immunofluorescence, sections were processed with MaxFluor^{TM} 488 Immunofluorescence Mouse Detection Kit (#MF31-M, Dianova, Hamburg, Germany) as per manufacturer's protocol and incubated 15 minutes with DAPI (1:1000 dilution in PBS, 1 mg/ml solution, #62248, Thermo Fisher Scientific, Massachusetts, USA). The rabbit/mouse EnVision-HRP/DAB+ kit (#K5007, Dako, CA, USA) was used for IHC, and sections were counterstained with haematoxylin.

Bright-Field Imaging, Confocal Imaging and MHC-II Quantification

All brightfield images were captured with Nikon E400 microscope, DS-Fil U2 camera, and NIS-Elements BR imaging software (Nikon Corporation, Tokyo, Japan) with 20x objective and processed with Fiji (46). Confocal fluorescence microscopy images were captured with 20x or 63x objective using a Zeiss LSM 880 Fast Airyscan Confocal microscope (Zeiss, Oberkochen, Germany). To minimize photobleaching, laser power typically was 20% under maximum, and the pinhole was set to 0.8–1.5. Images were processed with Zen Blue 2.6 software (Zeiss). Quantitative confocal image analyses of MHC-II staining in colonoids were performed using Fiji (46). The fluorescence intensity of MHC-II staining was determined by measuring the integrated density. Otsu's thresholding algorithm was applied to convert images with DAPI staining to binary images, and the nuclei (cell counts) were quantified using the Analyze Measure plugin in ImageJ. The corrected total cell fluorescence (CTCF) was calculated: CTCF = Integrated density-(Area of selection X Mean fluorescence of background readings). The average of CTCF/cell counts for five power fields per condition for each experiment is represented in the graphs. A total of 2000-6000 cells in the colonoids were counted per treatment condition. Quantification results are presented in **Supplementary File SF1**, sheet no 3.

Immunoblotting

Colonoids were taken from -80 °C, washed twice in ice-cold PBS before they were lysed for 2 hours on ice in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-4#0, 1 mM DTT, 1x Complete® EDTA-free protease inhibitor (#11873580001, Sigma-Aldrich), and 1x phosphatase inhibitor cocktail I (#P2850, Sigma-Aldrich) and III (#P0044, Sigma-Aldrich), respectively. Protein lysates were clarified by centrifugation at $14,000 \times g$ for 20 minutes at 4°C, and protein concentration was measured using the Bradford protein assay (Bio-Rad, California, USA). Protein lysates were denatured in $1 \times$ NuPage lithium dodecyl sulfate (LDS) sample buffer supplemented with 40 mM DTT for 10 minutes at 70°C before they were separated on 4–12% NuPage Bis-Tris gels (# NP0321BOX, Invitrogen, MA, USA) and electroblotted onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in Rockland Blocking Buffer for fluorescent Western Blotting (#MB-070, Rockland, PA, USA) for 1 hour at room temperature before incubation with the indicated antibodies overnight at 4°C. The blots were incubated with Dylight secondary antibodies (Invitrogen) for visualization. Images were obtained with LI-COR Odyssey and analyzed using Image Studio Software (LI-COR Biosciences, NE, USA). Total protein levels were normalized to GAPDH and expressed as fold change to untreated DMSO control. The following antibodies were used: HLA DR + DP + DQ mouse monoclonal antibody [CR3/43] (1:400 dilution, #ab7856, Abcam), HLA Class 1 ABC mouse monoclonal antibody [EMR8-5] (1:200 dilution, #ab70328 Abcam), STAT11 (9H2) mouse monoclonal antibody (1:1000 dilution, #9176, Cell Signaling Technology, Danvers MA, USA), STAT1 Y701:Phospho-Stat1 (Tyr701) (D4A7) rabbit monoclonal antibody (1:1000 dilution, #7649 Cell Signaling Technology), and GAPDH (D16H11) XP rabbit monoclonal antibody (1:5000 dilution, #5174, Cell Signaling Technology).

Quantification results are presented in **Supplementary File SF1**, sheet number 4.

Analysis of IFNγ Protein in Conditioned Medium

IFNγ measurement by ELISA was carried out using IFN gamma Human Uncoated ELISA Kit (#88-7316-86, Thermo Fischer Scientific) as per the manufacturer's instructions.

Statistical Analysis

Data from RNA sequencing was analyzed as described above. For all other datasets, statistical analyses were performed in GraphPad Prism 9.0. For normally distributed datasets containing two groups, paired T-test was used, and for normally distributed datasets with more than two groups, one-way ANOVA with Geisser-Greenhouse correction followed by Šidák multiple comparison testing was used. For data not following normal distribution, nonparametric Friedman's test followed by Dunn's multiple comparison tests were done. Comparisons between UC and non-IBD controls are made with Two-way ANOVA followed by Tukey's multiple comparison tests. All datasets with $P < 0.05$ was considered statistically significant and is indicated by *.

RESULTS

Differentiated Colonoids Express MHC-II Related Genes at Constitutive Conditions

MHC-II expression by IECs is particularly described in the small intestine, where it is expressed in differentiated cells at constitutive conditions (4) and in a subset of small intestinal undifferentiated epithelial stem cells (12). In colonic epithelium, it is considered to be absent in constitutive conditions and upregulated during inflammation (4, 16, 18). It is unclear if colonoids as a model system express MHC-II genes at constitutive conditions. Therefore, we characterized the expression of MHC-II related genes in differentiated and undifferentiated colonoids using an in-house RNA sequencing (RNA-seq) dataset from 3 non-IBD controls (D1-D3, **Table 1**). Although we noted large interindividual differences in normalized mRNA reads, we found that differentiated human colonoids expressed significantly higher levels of MHC-II related genes *HLA-DMA, HLA-DMB, HLA-DRA, HLA-DRB1*, and *HLA-DRB6* when compared to undifferentiated colonoids. Further, *HLA-DRB5, HLA-DQB1*, and the MHC-II transactivator *CIITA* and MHC-II invariant chain *CD74* had a tendency towards increased expression in differentiated epithelium compared to undifferentiated epithelium (**Figure 1B**). Since MHC-I gene expression is not well characterized in colonoids, we also explored its expression in differentiated vs. undifferentiated conditions. MHC-I gene subtype *HLA-C* was significantly upregulated in the differentiated epithelium, and other MHC-I related genes *HLA-A, HLA-B*, non-classical *HLA-E* and *HLA-F*, class I component Beta-2-Microglobulin (*B2M*) showed a similar tendency of increased expression in the differentiated epithelium compared to undifferentiated epithelium (**Figure 1C**). Taken together, differentiated colonoids express higher levels of MHC-II and MHC-I genes than undifferentiated colonoids.

TNF+Poly(I:C) Stimulation in Colonoids Enhances Expression of Genes Relevant to Antigen Presentation Pathway

Next, we looked at another in-house RNA-seq data set from colonoids derived from 3 non-IBD and 3 UC donors (D1-D3 and D7-D9, **Table 1**), stimulated with either TNF alone or in combination with Poly (I:C) (TNF+Poly(I:C)) for 24 hours. Pathway network analysis of the gene expression data revealed that IFN-dependent type I signaling pathway and IFNγ-dependent antigen presentation *via* the JAK/STAT pathways were the most upregulated pathways by the combined stimulation with TNF+Poly(I:C) (**Figure 2A**). Upon further examination of the IFNγ-dependent antigen presentation pathway (**Supplementary Figure 1A**), we observed no detectable levels of *IFNγ* gene expression. IFNγ receptors IFNGR1 and IFNGR2 were upregulated as well as Janus kinase *JAK1*, *JAK2*, and transcription factor *STAT1* were significantly upregulated (**Figure 2B**). Notably, *HLA-DQB1, HLA-DRA, HLA-DRB1, HLA-DR5*, and *CD74*, and MHC-I related genes *HLA-A, HLA-B, HLA-C, B2M*, non-classical genes *HLA-E* and *HLA-F*, were upregulated upon stimulation with the combination of TNF+Poly(I:C). Similar tendency for a subset of these genes was observed with TNF-stimulation alone when compared to unstimulated conditions (**Figure 2B**). Costimulatory *CD40* gene expression was also enhanced (**Figure 2B**), and a similar pattern of increased expression was observed for *CIITA* ($P=0.089$) in colonoids stimulated with TNF+Poly(I:C) (**Supplementary Figure 1B**). Since mucosa of UC patients has been shown to express costimulatory CD80 and CD86 that are important for T-cell activation, we looked at their expression in colonoids and found no expression in our RNA-seq data (data not shown). Several other genes relevant to antigen presentation pathway such as *CTSS, CD74, IRF1*, transcription factor *USF1*, transactivator of MHC-I *NLRC5*, and several proteosome subunits and transporter proteins, were upregulated in the colonoids stimulated with TNF+Poly(I:C) when compared to unstimulated controls (**Supplementary Figure 1C**). Together, TNF+Poly(I:C) stimulation of colonoids leads to enhanced expression of genes relevant to antigen presentation, including MHC-II expression. Interestingly, these effects were in line with upregulation of antigen presentation machinery observed in microdissected epithelium from IBD patients with active inflammation (**Supplementary Figure 1D**) (15).

Since MHC-II expression in intestinal epithelium is known to be induced by the IFNγ from the immune cells (19) and there are no immune cells present in our model, we next asked whether IFNγ could be secreted from the colonoids. We measured IFNγ in conditioned medium from unstimulated and TNF+poly(I:C) stimulated colonoids from $n = 5$ donors (D3, D5, D7, D9 and
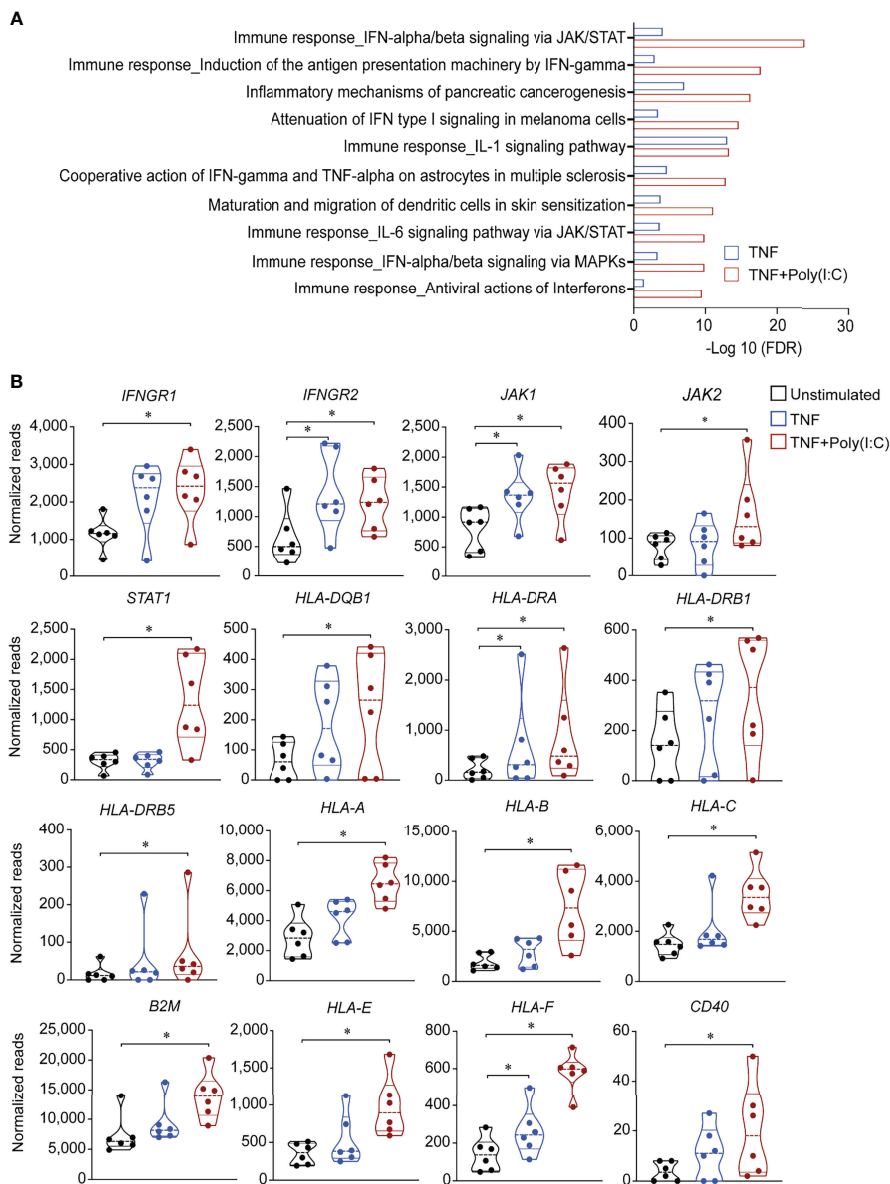
FIGURE 2 | Gene expression alterations in antigen presentation pathways due to TNF+Poly(I:C) stimulation of colonoids. Gene expression data from colonoids (n = 6 donors) stimulated with TNF alone (blue) or TNF+Poly(I:C) (red) for 24 hours in comparison with unstimulated (black) colonoids are depicted. **(A)** Enrichment analysis showing top 10 statistically significant (adjusted $P < 0.05$) differentially regulated pathways. Bars indicate –Log10 false discovery rate (FDR). Enrichment analyses are generated with MetaCore+MetaDrug™ version 21.3 build 70600. **(B)** Violin plots depicting normalized reads of genes relevant to antigen presentation pathways regulated by 24-hour stimulation with TNF or TNF+Poly(I:C) compared to unstimulated controls. *P* values are obtained by LIMMA linear models with least squares regression and empirical Bayes moderated t-statistics with Benjamini Hochberg FDR correction for multiple comparisons and * indicates $P < 0.05$.

D10, **Table 1**) by ELISA but could not detect any IFNγ protein (data not shown). This is in line with our findings that *IFNγ* mRNA was not detected in the colonoids.

TNF+Poly(I:C) Stimulation of Colonoids Induces Apical and Basolateral MHC-II Protein Expression

MHC-II protein expression and localization in IECs is altered during IBD, with enhanced expression observed at basolateral sides of the epithelium that may be functionally relevant for IECs to present antigens to immune cells in lamina propria (47). Therefore, we wanted to evaluate the protein expression and localization of MHC-II expression upon TNF+Poly(I:C) stimulation. Colonoids stimulated with TNF+Poly(I:C) or IFNγ for 24 hours were stained for MHC-II by immunofluorescence (**Figure 3A**) and immunohistochemistry (**Figure 3B**). In accordance with the gene expression data, we found that TNF+Poly(I:C) stimulation had a tendency towards

upregulation of MHC-II protein levels when compared to unstimulated colonoids (**Figure 3A**). Immunohistochemistry showed staining of differentiated colonoids from donor D2, confiring that this donor expressed MHC-II protein at untreated conditions (**Figure 3B**, left panels). The extent of MHC-II protein expression was enhanced upon stimulation with TNF+Poly(I:C) and IFNγ (**Figure 3B**, middle and right panels). In line with observation by others (19) the colonoids showed mainly enhanced apical expression of MHC-II after 24 hours of IFNγ stimulation. MHC-II protein appeared to be expressed in both basolateral and apical expression of MHC-II after 24 hours TNF+Poly(I:C)-stimulation (**Figure 3B**, middle panels). Basolateral and apical MHC-II expression was also observed in donors D2, D3, D7, and D8 by high resolution confocal imaging (**Supplementary Figure 2**). Thus, MHC-II protein expression can be induced directly by TNF+Poly(I:C) stimulation at basolateral sides as reported for epithelial MHC-II expression in active IBD (47).

Tofacitinib Downregulates TNF+Poly(I:C)-Dependent MHC-II Protein Expression

Gene expression data from colonoids indicated that upregulation of the MHC-I and MHC-II gene expression was associated with increased *JAK1/2* and *STAT1* expression (**Figure 2B**). Therefore, we investigated whether the pan-JAK inhibitor Tofacitinib could alter MHC-I and MHC-II expression. In comparison, we also investigated the effect of the anti-inflammatory glucocorticoid Budesonide on MHC-I and MHC-II expression. Colonoids from both non-IBD ($n = 4$) and UC ($n = 4$) donors (D3-D6 and D7-D10, **Table 1**) were pre-treated with 50 µM of Tofacitinib or 10 µM of Budesonide on day 11 in differentiation media and refreshed with new differentiation media and drugs on day 13 and one-hour before stimulation with TNF+Poly(I:C) on day 14. On day 15, 24-hours post-stimulation (**Figure 1A**), colonoids were collected and assessed for MHC-II and MHC-I expression by immunoblotting (**Table 1**). Colonoids treated with TNF+Poly (I:C) showed enhanced levels of MHC-II protein (**Figures 4A, B**)
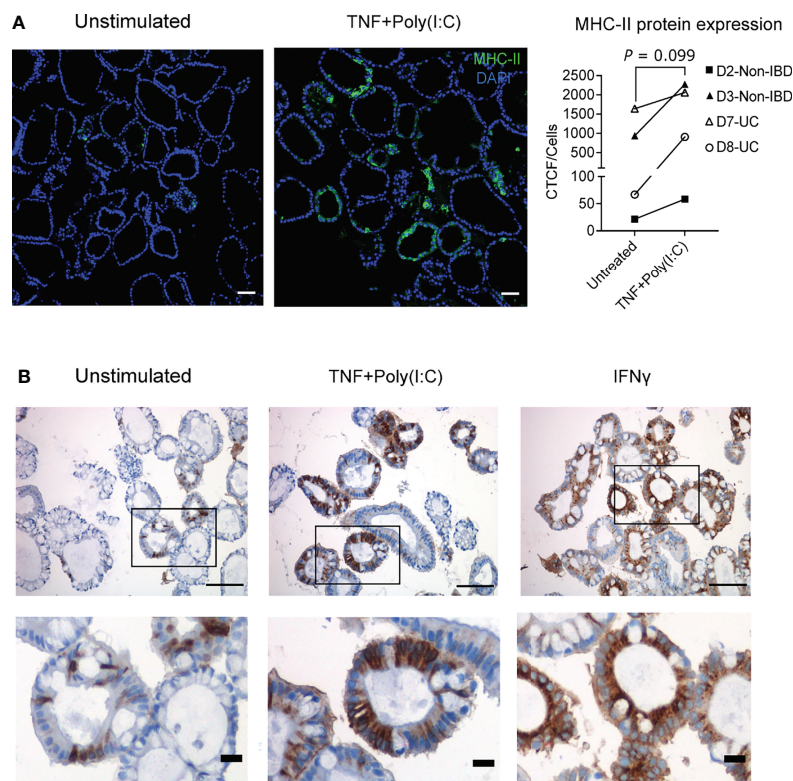
FIGURE 3 | TNF+Poly(I:C)-dependent MHC-II protein expression and localization in colonoids. **(A)** Ligand induced MHC-II protein expression was confirmed by immunofluorescence staining with colonoids from 2 non-IBD (D2-D3) and 2UC (D7-D8) donors. For non-IBD donor D2, this was reproduced in two independent experiments. Representative images are shown on the left. Nuclei are stained blue (DAPI), green colour shows MHC-II protein expression. MHC-II protein quantification as indicated by CTCF/cell counts per condition, for each experiment (averaged counts for D2). Images are obtained with 20x magnification and scale bars indicate 100 µm. *P* values are obtained by paired T-test. **(B)** Immunohistochemistry staining of MHC-II protein localization shown by brown colour in unstimulated colonoids (left), colonoids stimulated with TNF+Poly(I:C) (middle) and IFNγ (right). The colonoids were derived from non-IBD control D2 (representative images from $n = 3$ experiments). Demarcated areas in top row are shown magnified in picture below. Images are obtained with 20x magnification and scale bars indicate 100 µm in the top row and 20 µm in the bottom row.
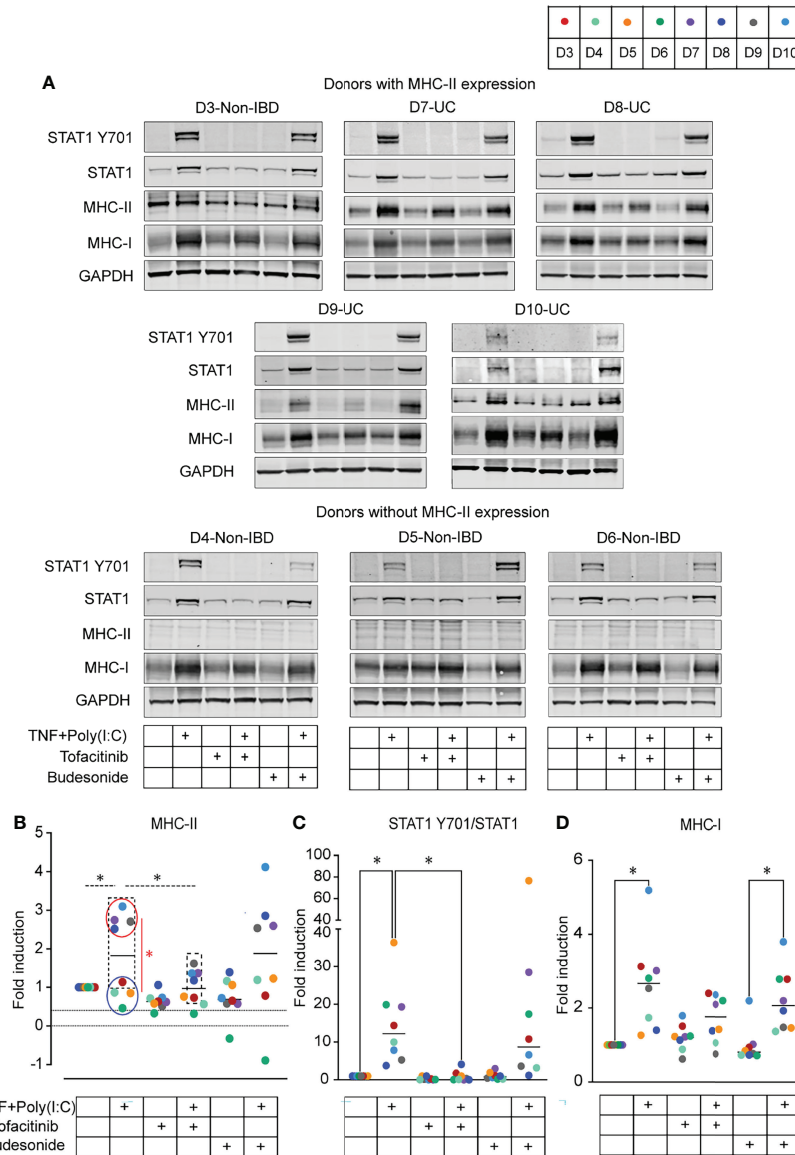
FIGURE 4 | Effect of Tofacitinib and Budesonide pre-treatment on TNF+Poly(I:C)-stimulation in colonoids by immunoblotting. **(A)** Immunoblots from colonoids showing STAT1 Y701, STAT1, MHC-II, MHC-I protein expression in colonoids from donors expressing MHC-II (top) and donors without MHC-II expression (bottom). Immunoblotting quantification of MHC-II protein expression **(B)**, activated STAT1 protein expression represented as STAT1 Y701/STAT1 **(C)**, and MHC-I protein expression **(D)**. Fold expression is generated by normalizing to DMSO unstimulated control for each donor and further normalized to GAPDH expression. In all quantification results, each donor is indicated by colored dots, and donor numbers and colour codes are shown at the top of the figure. Dashed lines represent statistical tests on donors expressing MHC-II at a constitutive level (D3, D7-D10, marked by dashed box). Colonoids from UC donors (D7-D10, red circle) are compared with non-IBD donors (D3-D6, blue circle) and red line represents statistical testing between these groups. P values are obtained by one-way ANOVA followed by Šídák's multiple comparisons test (B-dashed lines), Two-way ANOVA followed by Šídák's multiple comparisons (B-red line) or nonparametric Friedman's test followed by Dunn´s multiple comparison tests **(C, D)**. * indicates $P < 0.05$. Data represented from UC donor D7 are averaged from two independent experiments.

in donors with MHC-II expression at constitutive conditions (D3, D7-D10, dashed boxes), verifying gene expression data (**Figure 2B**). In donors lacking MHC-II expression at constitutive conditions (D4-D6), we did not observe enhanced TNF+Poly(I:C)-dependent MHC-II protein expression. There was a significant upregulation of MHC-II protein expression in TNF+Poly(I:C) stimulated colonoids derived from UC donors

(red circle) when compared to non-IBD donors (blue circle) (**Figure 4B**). Treatment with TNF+Poly(I:C) in colonoids was also associated with increased activation of STAT1 protein in all donors (**Figure 4C**), in line with the significant upregulation of STAT1 mRNA detected by RNA-seq (**Figure 2B**). Tofacitinib pre-treatment inhibited phosphorylation of STAT1 and reduced the expression of total STAT1 protein levels in colonoids treated

with TNF+Poly(I:C) (**Figures 4A, C**). Importantly, Tofacitinib significantly down-regulated expression of TNF+Poly(I:C)-induced MHC-II protein levels in those donors that had MHC-II expression (D3, D7-D10, dashed box) by immunoblotting (**Figures 4A, B**). Budesonide pre-treatment in colonoids neither influenced the TNF+Poly(I:C)-depedent activation of STAT1 protein nor MHC-II protein expression. We also observed TNF+Poly(I:C)-dependent increase in MHC-I protein levels when stained with a pan MHC-I antibody recognizing HLA-A, HLA-B and HLA-C (**Figures 4A**, **Figure 4D**), verifying gene expression data (**Figure 2B**). Some donors showed a tendency towards downregulation of TNF+Poly(I:C)-depedent MHC-I protein levels due to Tofacitinib pre-teatment, but this was not statistically significant (**Figure 4D**). Further, Budesonide pre-treatment did not have any effect on TNF+Poly(I:C)-depedent MHC-I protein levels. Thus, TNF+Poly(I:C)-dependent increase of MHC-II protein expression in colonoids was downregulated with Tofacitinib pre-treatment possibly by repressing activation of STAT1, while pre-treatment with Budesonide had no effect.

Next, we investigated whether Tofacitinib and Budesonide pre-treatment influence TNF+Poly(I:C)-dependent protein localization of MHC-II. We performed endpoint immunofluorescence staining with the experimental setup described in **Figure 1A**, using a new passage of colonoids from the same donors used for endpoint immunoblotting analysis (**Figure 4**). Tofacitinib pre-treatment downregulated and Budesonide pre-treatment did not affect TNF+Poly(I:C)-dependent MHC-II protein expression (**Figure 5A**) in those donors where MHC-II expression was observed (D3, D7, D8, and D10, dashed box), in line with immunoblotting results (**Figures 4A, B**). We observed apical and basolateral expression of TNF+Poly(I:C)-dependent MHC-II protein expression, as shown in **Figure 3**, but did not find any alterations in protein localization due to the drug treatments.

Interindividual Differences in *In Vivo* MHC-II Protein Expression Are Mimicked in Colonoids

In our endpoint immunoblotting (**Figure 4**) and immunofluorescense (**Figure 5**) experiments we noticed that MHC-II protein was expressed in colonoids from all UC donors (D7-D10) and one non-IBD donor (D3). Colonoids from the non-IBD donors D4-D6 did not have any MHC-II protein expression (**Figure 4A**). In order to better understand the interindividual differences in MHC-II expression, we examined whether the presence of MHC-II expression in differentiated colonoids mimicked *in vivo* MHC-II expression in colonic epithelium from the same donors (**Table 1**). Amongst the 6 donors compared (4 non-IBD and 2 UC), we observed that colonoids from 1 non-IBD donor (D3) and 2 UC donors (D7 and D8) with MHC-II protein expression in colonoids (**Figure 4**) mimicked MHC-II expression in the colonic epithelium *in vivo* (**Figure 5B**). Further, donors D4-D6 that did not express MHC-II in colonoids also lacked MHC-II expression in the colonic epithelium *in vivo* (**Figure 5B**). Thus, the reproducible interindividual differences in MHC-II expression observed in

colonoid experiments recapitulate *in vivo* epithelial MHC-II expression.

DISCUSSION

In the current study using non-IBD and UC-patient-derived colonoids, we have demonstrated that IBD-associated pro-inflammatory signals TNF and dsRNA (Poly(I:C)) together upregulate MHC-II expression in differentiated colonic epithelium in a pattern similar to that seen in IBD epithelium (15). Importantly, we provide insight into the role of Tofacitinib in downregulating TNF+Poly (I:C) induced MHC-II expression.

Tofacitinib is approved for treatment of ulcerative colitis in patients where first and second line treatments have failed (31, 32). However, the effects of Tofacitinib on IECs remain largely unexplored. The cell permeable small molecule Tofacitinib (CP-690550) Citrate used in our experiments inhibits JAK3 and JAK1 and to a lesser extent JAK2. A decade ago it was shown that CP-690550 reduced TNF-induced synthesis of chemokines like MCP1(CCL2), IP-10 (CXCL10) and RANTES (CCL5) in a selective manner *via* the JAK/STAT signalling pathway since TNF-induced IL-8 (CXCL8) synthesis and secretion remained unchanged (48). Because stimulation of colonoids with TNF+Poly(I:C) was associated with enhanced *JAK1/2* and *STAT1* gene expression, it was of interest to examine the effect of Tofacitinib on the regulation of MHC-II and MHC-I. Tofacitinib has previously been described to regulate processes related to antigen presentation in non-intestinal epithelial cells. It was shown to downregulate IFNγ-induced MHC-I expression in non-small cell lung cancer cell lines (49), and CD80/CD86 in dendritic cells stimulated with lipopolysaccharide, thereby reducing T-cell stimulatory capacity (36). The latter study, however, also showed an unaltered *HLA-DR* expression in DCs upon treatment with Tofacitinib (36). In our dataset, Tofacitinib treatment downregulated TNF+Poly(I:C)-dependent MHC-II protein expression in colonoids. The glucocorticoid Budesonide had no effect on TNF+Poly(I:C)-dependent MHC-II expression, suggesting a significant involvement of JAK1/2-STAT1 signaling pathway in the regulation of MHC-II expression in colonic epithelium. While Tofacitinib has not been directly implicated in the regulation of MHC-II, JAK1/2 inhibitor Baricitinib, a drug approved for Rheumatoid arthritis, has been shown to modulate MHC-II on allogenic antigen-presenting cells and prevent graft vs host disease (50).

Increased MHC-II expression in IECs during active inflammation is considered to be secondary to IFN-γ stimulation from intraepithelial lymphocytes or lamina propria immune cells (19–22). These cell types are not present in our intestinal epithelial organoid model system, and we did not detect IFNγ expression or secretion in our colonoids that could indicate an autocrine loop. We did show that Tofacitinib pre-treatment inhibited phosphorylation of STAT1 and reduced the expression of total STAT1 protein levels in colonoids treated with TNF+Poly(I:C). One possibility is that TNF+Poly(I:C) stimulate the secretion of type I IFNs that can activate the JAK/STAT pathway in an autocrine faschion (28, 48).
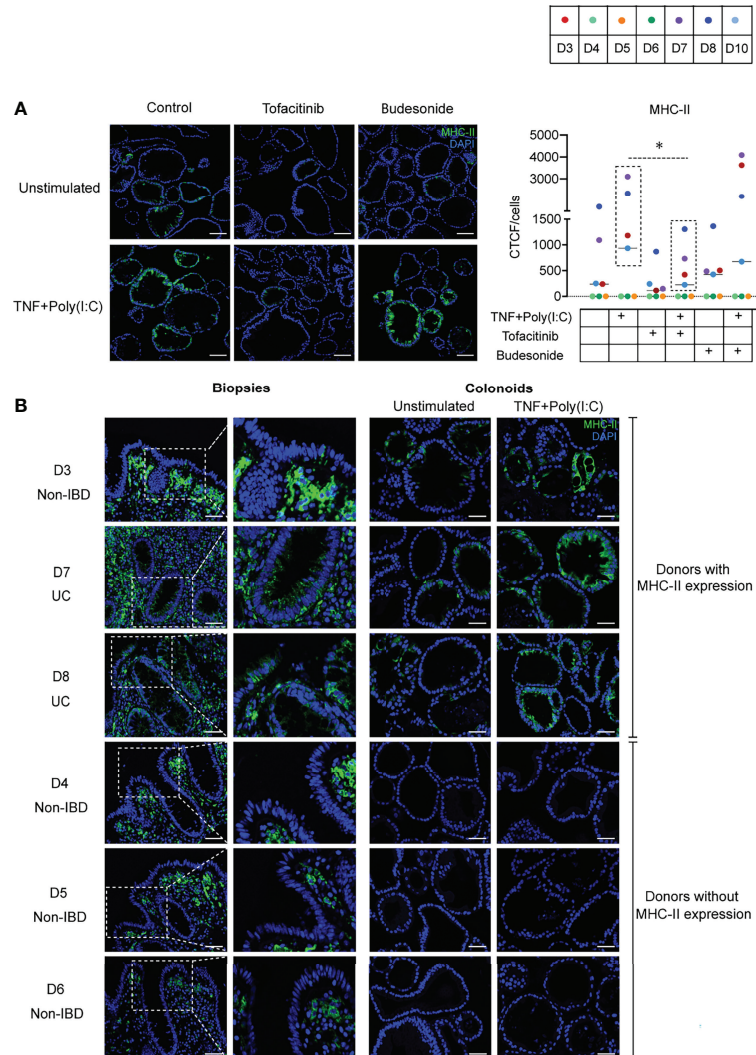
FIGURE 5 | MHC-II localization due to Tofacitinib and Budesonide pre-treatment and comparison with *in vivo* epithelial MHC-II expression. **(A)** Effects of ligand and drug treatment on MHC-II protein expression in colonoids detected by immunofluorescence staining ($n = 8$ experiments derived from 7 donors, for UC donor D8, data represented are averaged from two independent experiments). Representative images are shown on the left. Nuclei are stained blue (DAPI), green colour shows MHC-II protein expression. MHC-II quantification as indicated by CTCF/cell counts per condition for each experiment are shown on the right. Images are obtained with 20x magnification, scale bars indicate 100 μm. In quantification result, each donor is indicated by coloured dots and donor numbers and colour codes are is shown at the top of the figure. As in **Figure 4B**, the dashed lines indicate that TNF+Poly(I:C) induced MHC-II protein expression was significantly downregulated by Tofacitinib in donors that constitutively express MHC-II (D3, D7, D8, and D10, in dashed box). *P* values are calculated by nonparametric Friedman's test followed by Dunn's multiple comparison tests and * indicates $P < 0.05$. **(B)** MHC-II protein expression as represented by immunofluorescence staining in *in vivo* biopsies (left panel) and corresponding unstimulated and TNF+Poly(I:C)-stimulated colonoids (right panel) from non-IBD donors (D3-D6) and UC-donors (D7-D8). Areas in the biopsies marked in white dotted boxes are shown adjacent with higher magnification. *In vivo* colonic biopsies for immunostaining from the UC patiens are taken from inflamed area. MHC-II expression is shown in green and DAPI in blue. Scale bar represents 50 μm.

However, to our knowledge type I IFNs have not been reported to activate MHC-II expression in IECs, and we have not found IFNβ induced protein expression of MHC-II in colonoids (data not shown), as demonstrated for IFNγ. Thus, more studies are needed to define the exact signalling pathways involved in TNF+Poly(I:C) induced MHC-II expression.

Whether or not enhanced antigen presentation in IBD is deleterious or protective is unknown. However, since antigen presentation is a central process in adaptive immunity, the impact of its regulation by Tofacitinib in IBD should be evaluated. Our data indicate that colonoids from donors that expressed MHC-II genes at constitutive conditions showed increased expression in differentiated versus undifferentiated conditions. This is in accordance with data presented by Kelson et al., who also reported *HLA-DR* expression in colonoids derived from pediatric IBD patients (21) and Wosen

et al. (19), who showed that differentiated small intestinal organoids express MHC-II in response to IFNγ. Our data did not show a high expression of MHC-II genes in undifferentiated conditions, in contrast to studies observed by Biton M et al. (12), who demonstrated that undifferentiated murine intestinal stem cells express MHC-II. The differentiation protocol used in our study generates organoids containing polarized cells of all the major colonic cell types including absorptive cells, goblet cells and enteroendocrine cells (28). Immunostaining for MHC-II showed that some colonoids expressed MHC-II while some did not. One explanation can be that the there were more fully differentiated cells in the colonoids that expressed most MHC-II, since we showed that differentiated cells express more MHC-II than undifferentiated cells. Interestingly, colonoids from UC donors appeared to have more MHC-II positive cells than colonoids from non-IBD donors, but we need substantially more donors in each group before we can separate between general interindividual differences and differences that are disease specific. Further characterization of MHC-II in specific colonic epithelial subtypes is required for a better understanding of the functionality in colonic epithelium. Studies of interindividual differences in cell composition will also be important to understand the donor heterogeneity observed.

The RNA-seq data demonstrated that differentiated colonoids induce some of the genes in the antigen presentation pathway when stimulated with TNF. Colonoids stimulated with a combination of TNF and Poly(I:C) had upregulation of *IFNGR1* and *IFNGR2*, *JAK1/2* kinases, transcription factor *STAT1* and *NLRC5*, costimulatory genes *CD74* and *CD40*, and several transporter proteins that are relevant to both MHC-II and MHC-I dependent antigen presentation. Importantly, these findings are also captured in microdissected epithelium from IBD patients with active disease (**Supplementary Figure 1D**) (15). Therefore, TNF+Poly(I:C)-dependent expression of antigen presentation pathway may provide a conceptual basis for enhanced antigen presentation involving signaling from DAMPs or viral infections in the context of TNF-driven inflammation during active IBD.

Localization of MHC-II in IECs was shown to be relevant for IBD pathogenesis. MHC-II expression is enhanced at the basolateral sides of the epithelium during active IBD, presenting antigen to the immune cells in lamina propria to facilitate adaptive immunity during inflammation (5, 47). Previous studies show that MHC-II was expressed on the apical side of enteroids stimulated with IFNγ for 24 hours. The authors also showed that long-term stimulation with IFNγ for 72 hours induced basolateral expression of MHC-II (19). Our results however demonstrate that stimulation of colonoids with TNF+Poly(I:C) leads to both apical and basolateral expression of MHC-II already after 24 hours. Apical MHC-II could be important for directly controlling microbiome composition in the colon (38, 51, 52) as well as activation and differentiation of intraepithelial lymphocytes (14). Basolateral MHC-II may be essential for enhancing MHC-II expression on mononuclear phagocytes (38) or presenting to CD4+ T cells in the lamina

propria (12, 24). Therefore, apical and basolateral MHC-II may have multiple roles during adaptive immunity in IBD. While the focus of our study is to understand regulation in colonic epithelium, a limitation is that the colonoid model system does not allow for evaluating the functionality of enhanced IEC-specific TNF+Poly(I:C)-dependent MHC-II as well as its regulation by Tofacitinib in immune cells. However, future colonoid-immune co-culture studies may be adopted to address these limitations.

In the present work we performed a series of independent experiments to examine expression of MHC-II with several techniques (RNAseq, Western blot, IHC and Confocal imaging) in colonoids derived from totally ten donors (**Table 1**). We observed that colonoids derived from four UC-patients (D7-D10) expressed MHC-II in all the independent experiments. Thus, we could detect MHC-II expression with different techniques, that was also reproducible in independent experiments using the same detection techniques. Amongst the six non-IBD donors included, two expressed MHC-II (D2 and D3), while four did not (D1, D4-D7). Thus, we conclude that MHC-II expression in intestinal epithelial cells appeared donor-dependent. Importantly, the presence or absence of MHC-II protein in differentiated colonoids could be captured *in vivo* in the colonic epithelium of corresponding donors, showing a major strength of the colonoid model system. In two out of six non-IBD controls and all four UC patients used in this study, we found variable MHC-II expression at constitutive conditions. Donor-specific variation in MHC-II protein expression has also been reported previously (19). Constitutive MHC-II protein expression in colonoids was upregulated with TNF+Poly(I:C) stimulation which was associated with enhanced STAT1 activation. STAT1 activation was also observed in colonoids from those donors that lacked MHC-II expression, after TNF+Poly(I:C) stimulation. Thus, colonoids from all donors responded to TNF+Poly(I:C). MHC-II genes are amongst the most hyper polymorphic genes and are influenced by environmental factors such as microbiome (14, 24, 51) and diet (25). Therefore, the variability in MHC-II protein expression observed in our study could be due to epigenetics, gene variations or other undisclosed factors in the donors. In 2016, Dotti et al. (53) showed that some gene expression differences are maintained in *ex vivo* expanded epithelial organoid cultures generated from biopsy samples of patients with UC compared with non-IBD subjects. The authors concluded that these differences are possibly due to permanent changes in the stem cell compartment and speculated whether epigenetic changes may contribute to expression alterations of the UC epithelium. In future studies it will be interesting to study wether e.g., epigenetic changes triggered in inflamed tissue persist during culture and participate to the interindividual variations in MHC-II expression observed. We also observed donor-dependent variability to Tofacitinib treatment. These results highlight the need for understanding interindividual differences in disease pathobiology and drug responses, which may be achieved by utilizing patient-derived colonoid model systems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. A subset of the data are available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE172404.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Norway Regional Committee for Medical and Health Research Ethics (reference numbers 5.2007.910 and 2013/212/REKmidt). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TB supervised the study. SG, MDH, HKS, IAR, AvBG, IB, TB contributed to experimental design, generated, and analyzed data. AEØ and AKS collected and characterized patient samples. SG made figure panels and drafted the manuscript along with TB. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.882277/full#supplementary-material

Supplementary Figure 1 | Regulation of antigen presentation genes in colonoids and microdissected epithelium. Gene expression data from colonoids (n = 6 donors) stimulated with TNF alone or TNF+Poly(I:C) for 24 hours in comparison with unstimulated colonoids. **(A)** Canonical pathway map generated by MetaCore+MetaDrug™ version 21.3 build 70600 software representing antigen presentation pathway as one of the top pathways regulated by TNF+Poly(I:C) stimulation in colonoids. Bars with red indicate upregulated expression of indicated genes by 1:TNF stimulation or 2:TNF+Poly(I:C) stimulation. **(B)** Violin plots depicting *CIITA* gene expression in colonoids. **(C)** Heatmap depicting log2 ratio of genes relevant to the antigen presentation pathway in TNF or TNF+Poly(I:C)-stimulated colonoids when compared to unstimulated controls. **(D)** Heatmap depicting log2 ratio of genes in microdissected colonic epithelium from active IBD (n = 12) compared to uninflamed epithelium (healthy controls and IBD in remission, n = 17). Part of this data is also presented in Sæterstad et al. (15),. *P* values are obtained by LIMMA linear models with least squares regression and empirical Bayes moderated t-statistics with Benjamini Hochberg FDR correction for multiple comparisons and * indicates *P* < 0.05.

Supplementary Figure 2 | MHC-II localization in colonoids stimulated with TNF+Poly (I:C). Immunofluorescence staining of MHC-II protein in IFNγ or TNF+Poly(I:C) treated colonoids. Nuclei are staining blue (DAPI), green shows MHC-II protein expression in the colonoids which are derived from four donors (D2, D3, D7 and D8,). Confocal fluorescence microscopy images were captured with 20x and 63x objective. On left images with 20x magnification and scale bar 100 μm. White arrows indicate organoids that are zoomed and presented in the images on the right. Zoomed images on the right are from 20x magnification for IFNγ stimulated colonoids and 63x magnification for TNF+Poly(I:C) stimulated colonoids and scale bars indicate 50 μm.

Supporting Information | (Dropbox) Dropbox link: https://www.dropbox.com/sh/a86zh9ocfmlqgi4/AADN3bx8yVwDAL-XV0RPNRX5a?dl=0.

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Case Report: Successful Treatment of Alopecia Universalis With Tofacitinib and Increased Cytokine Levels: Normal Therapeutic Reaction or Danger Signal?

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Alopecia universalis (AU) is an autoimmune disorder characterized by non-scarring hair loss in the scalp, eyebrows, beard, and nearly the entire body, negatively affecting patient prognosis. Available treatments are usually unsatisfactory. The autoimmune attacks of hair follicles induced by CD8+ T cells and the collapse of hair follicle immune privilege are believed to be the leading causes of AU. Additionally, interferon (IFN)-γ plays an important role in triggering the collapse of hair follicle immune privilege and impairing hair follicle stem cells. Furthermore, the upregulation of Janus kinase (JAK)3 and phospho-signal transducer and activator of transcription (pSTAT)3/STAT1 in alopecia areata patients suggest that JAK inhibitors can be a potentially promising choice for AU patients for the reason that JAK inhibitors can interfere with JAK-STAT signaling pathways and inhibit IFN-γ. Herein, we report a case of AU successfully treated with tofacitinib. However, this beneficial response in the patient was accompanied by a remarkable increase in peripheral blood cytokine levels during tofacitinib treatment.

Keywords: alopecia universalis, tofacitinib, hair loss, Janus kinase inhibitor, cytokines

INTRODUCTION

Alopecia universalis (AU) is an autoimmune disorder characterized by non-scarring hair loss in the scalp, eyebrows, and beard, potentially resulting in complete hair loss all over the body. AU's treatment options are usually unsatisfactory, and no specific effective therapies are available (1). The exact etiopathogenesis is unclear, but the collapse of the immune system and autoimmune attack of hair follicles induced by CD8+ T cells are believed to be the leading causes of AU (2). Gene expression of inflammatory markers (interleukin [IL]-2, Janus kinase [JAK]3, and IL-15), T helper type (Th) 1 pathway cytokines (interferon [IFN]-γ), and Th2 pathway cytokines (IL-13) increase in the lesional scalp of alopecia areata (AA) patients. Meanwhile, phospho-signal transducer and activator of transcription (pSTAT)3/STAT1 is also upregulated (3, 4). The serum cytokine level of IFN-γ is also significantly increased (5), suggesting that JAK inhibitors can be a potentially beneficial choice for AU patients due to JAK inhibitors interfering with the JAK-STAT signaling pathway and

inhibiting IFN-γ. Herein, we report a case of AU successfully treated with tofacitinib. However, a remarkable increase in peripheral blood cytokine levels following tofacitinib treatment in the AU patient was observed.

CASE DESCRIPTION

A 53-year-old man visited our clinic complaining of quickly worsening hair loss in the scalp, eyebrows, beard, armpit hair, groin, and nearly his entire body 5 months ago, seriously affecting his appearance and increasing his psychological burden. Other than these outcomes, he was healthy without any medical history or family history of similar diseases except for transient urticaria 3 months ago. No previous treatments were administered to the patient. Skin examination showed hair loss in the scalp, eyebrows, beard, and the rest of his body (**Figure 1**). The Severity of Alopecia Tool (SALT) score (6) and the Alopecia Areata Investigator Global Assessment (AA-IGA[TM]) score (7) was used to assess his hair loss. His SALT score was 100, and AA-IGA[TM] score was 4, which suggested that his condition was severe. Routine analysis of hemogram, hepatic and renal function, corticosteroid hormone, serum IgE, and T-SPOT.TB tests (T-SPOT) were all normal. We also examined his peripheral blood T-cell subsets, which showed that CD3+ T cells, CD3+CD4+ T cells, and CD4+/CD8+ T cells were normal except for CD3+CD8+ T cells, which were slightly increased. Simultaneously, peripheral blood cytokines were examined using a commercial multiple cytokine detection kit [multiple microsphere flow cytometry] (qdraisecare, China) to examine the cytokine level. The normal values of the cytokine level were defined based on the serum cytokine level of 198 healthy people. Results showed that IL-6, IL-17, and IL12p70 increased, while IL-4, IL-10, IFN-γ, and tumor necrosis factor-α (TNF-α) levels were within the normal range (**Figure 2**). According to the typical clinical manifestation and medical history, the patient was diagnosed with AU.

After written informed consent was obtained, the patient was administered 5 mg of oral tofacitinib daily. Fortunately, a rapid and significant therapeutic effect was observed after the treatment, and his hair regrew gradually, although the color of the hair was white. No systemic side effects were found after treatment except slightly elevated glutamic-pyruvic transaminase (ALT) levels and blood lipid levels. Twenty-four weeks after tofacitinib treatment, the SALT and AA-IGA[TM] scores decreased significantly to zero (**Figure 2**). We reexamined his peripheral blood T-cell subsets and found these values returned to normal. However, his peripheral blood cytokines were significantly increased, including Th1 cytokines IFN-γ, TNF-α, IL12p70, Th2 cytokines IL-4, IL-6, IL-10, and Th17 cytokines IL-17. IFN-γ was found to have increased 72 times compared with the baseline before tofacitinib treatment (**Figure 2**). Considering the patient's safety and benefits observed with the patient's regrown hair, oral tofacitinib was discontinued, and Diprospan (7 mg/ml, 1 ml, contains betamethasone disodium phosphate 2 mg and betamethasone dipropionate 5 mg) intramuscular injections were administered once every 4 weeks to sustain the effect. Twenty-four weeks after Diprospan treatment, his hair growth was maintained. We reexamined his peripheral blood cytokines and found that all the cytokines decreased to normal, with only IL12p70 remaining slightly above the standard level. However, this value still decreased compared to the baseline (**Figure 2**). The patient was very satisfied with both tofacitinib and Diprospan treatment, and the frequency of the Diprospan treatment was decreased gradually. No adverse effects were reported during the treatment or the follow-up.

DISCUSSION

AU is the most severe autoimmune disease affecting hair follicles of the scalp and the whole body and is mediated by CD8+ T cells (8). However, its exact pathogenesis remains unclear. Complex immunology, genetics, epigenetics, various environmental factors, and oxidative stress are suggested to participate in the development of the disease (9), especially as it relates to the collapse of hair follicle immune privilege, which is believed to be the leading cause (2). Current traditional treatments, including corticosteroids, immunosuppressive agents, topical minoxidil,

FIGURE 1 | The clinical manifestation of the patient's hair after tofacitinib treatment; at 8, 16, and 24 weeks, the hair has regrown gradually.
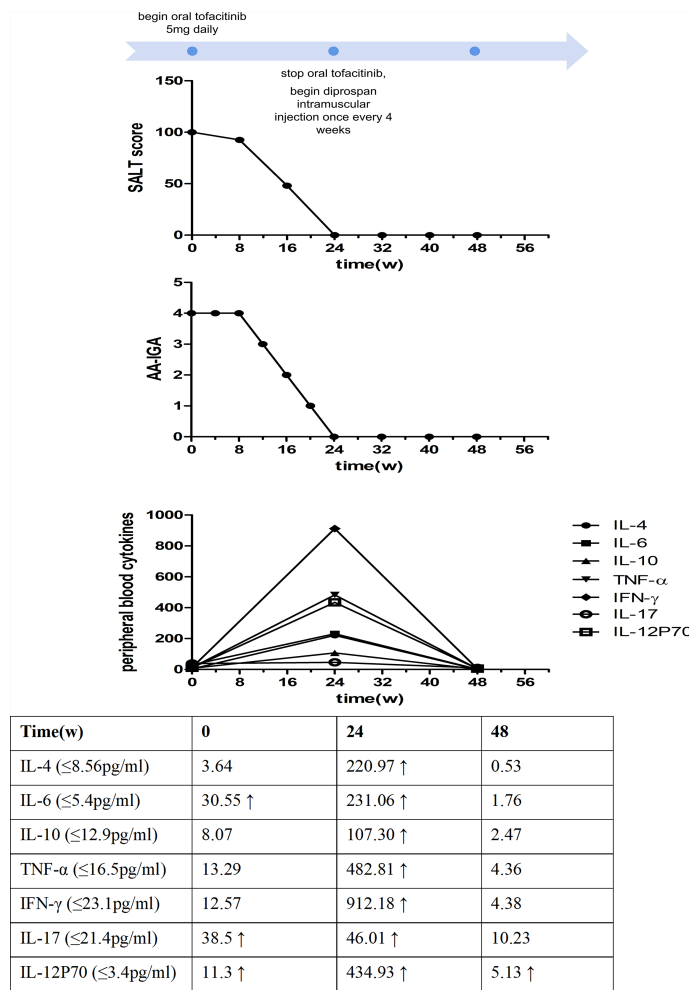
FIGURE 2 | Time course of the treatment and changes in the Severity of Alopecia Tool (SALT) score, the Alopecia Areata Investigator Global Assessment (AA-IGA[TM]), and the levels of peripheral blood cytokines.

Time(w)	0	24	48
IL-4 (≤8.56pg/ml)	3.64	220.97 ↑	0.53
IL-6 (≤5.4pg/ml)	30.55 ↑	231.06 ↑	1.76
IL-10 (≤12.9pg/ml)	8.07	107.30 ↑	2.47
TNF-α (≤16.5pg/ml)	13.29	482.81 ↑	4.36
IFN-γ (≤23.1pg/ml)	12.57	912.18 ↑	4.38
IL-17 (≤21.4pg/ml)	38.5 ↑	46.01 ↑	10.23
IL-12P70 (≤3.4pg/ml)	11.3 ↑	434.93 ↑	5.13 ↑

and contact immunotherapy, show limited effects with adverse reactions. Recent studies have suggested that JAK inhibitors could be a promising treatment option for inflammatory diseases (1). Specifically, they can interfere with T cell-mediated inflammatory signaling pathways to mediate these outcomes. Tofacitinib, a JAK inhibitor, mainly acts on JAK 1 and 3 and has been previously investigated in AA treatment. These studies show that it is well-tolerated and effective for severe and recalcitrant cases without any reported serious adverse effects, although tofacitinib is unable to maintain a durable response when treatment is stopped (10). After tofacitinib treatment, our patient's hair regrew gradually, and the SALT and AA-IGA[TM] scores decreased significantly (**Figure 2**). The beneficial response to regrowth of our patient's hair suggests that tofacitinib can be an optimal choice for AU.

Notably, the significant increase in cytokine levels in peripheral blood may be of concern. Due to the potential of inducing other unknown adverse reactions, we discontinued

tofacitinib following the treatment period. Cytokine levels decreased after discontinuation, suggesting that tofacitinib increased cytokine levels. Studies have shown that Th1, Th2, and Th17 cells may contribute to AA development (11), which is consistent with our findings. Serum Th1 cytokines, including IFN-γ, TNF-α, and IL-12p70, and serum Th2 cytokines IL-4, IL-6, IL-10, and Th17 cytokines IL-17 participated in the progression of AU. Tofacitinib can inhibit cytokines in hair follicles of AA patients, including IL-2, IL-4, IL-7, IL-15, IL-21, and IFN-γ, by blocking the STAT phosphorylation and disrupting the signaling pathway of JAK 1/3 (10). However, studies on peripheral blood cytokine changes after tofacitinib treatment are still lacking. Only one study evaluated serum IL-2, IL-4, IL-15, and IL-17 in patients with AA, in which all were observed to decrease after tofacitinib treatment (12), which was not consistent with our results. To our knowledge, no studies on changes in cytokine levels before and after tofacitinib treatment in AU patients have been reported. Our case is the first one

exploring the cytokine level change of tofacitinib treatment in a patient with AU. However, because this study involved only one patient, we are limited by the small sample value. Whether there are any different reactions to tofacitinib between AA and AU requires further clarification and larger sample populations to investigate its reliability.

Studies on mouse models demonstrated that IFN-γ, a JAK-STAT (signal transducer and activator)-dependent cytokine, was crucial for the development of AA. Intravenous injection of IFN-γ could induce AA-like hair loss in C3H/HeJ mice (13). As previously demonstrated, JAK inhibitors interfere with JAK-STAT signaling pathways and inhibit IFN-γ (8). Therefore, oral tofacitinib could prevent and treat AA *via* these mechanisms. However, our case's peripheral blood IFN-γ level increased significantly during hair regrowth after tofacitinib treatment. Therefore, the exact pathogenesis of cytokine level increase and whether it is a normal therapeutic reaction or a potential safety concern have not been comprehensively elucidated and requires further investigation.

CONCLUSION

Overall, tofacitinib has shown a remarkable effect in treating AU, which offers a potentially optimal choice for AU patients. However, we still need to evaluate its safety and determine why cytokine levels increase and whether it will adversely affect the patient. Further studies and more extensive population research are needed to support its long-term efficacy and safety for the potential treatment of AU.

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 Henan Provincial People's Hospital Medical Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LY and HY researched the data, contributed to the discussion, wrote the manuscript, and reviewed the manuscript. SZ and YH researched the data and contributed to the discussion. SMZ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Baricitinib therapy response in rheumatoid arthritis patients associates to STAT1 phosphorylation in monocytes

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Baricitinib is a Janus kinase (JAK) 1 and 2 inhibitor approved for treating rheumatoid arthritis (RA). The JAK/STAT system is essential in the intracellular signaling of different cytokines and in the activation process of the monocyte lineage. This study verifies the effects of baricitinib on STAT phosphorylation in monocytes of RA patients and evaluates the correlation between STAT phosphorylation and response to therapy. We evaluated the disease activity of patients (DAS28CRP) at baseline (T0) and after 4 and 12 weeks (T1–T3) of treatment with baricitinib, dividing them into responders (n = 7) and non-responders (n = 7) based on the reduction of DAS28CRP between T0 and T1 of at least 1.2 points. Through flow cytometry, STAT1 phosphorylation was analyzed at T0/T1/T3 in monocytes, at basal conditions and after IL2, IFNα, and IL6 stimulation. We showed that monocyte frequency decreased from T0 to T1 only in responders. Regarding the phosphorylation of STAT1, we observed a tendency for higher basal pSTAT1 in monocytes of non-responder patients and, after 4 weeks, a significant reduction of cytokine-induced pSTAT1 in monocytes of responders compared with non-responders. The single IFNα stimulation only partially recapitulated the differences in STAT1 phosphorylation between the two patient subgroups. Finally, responders showed an increased IFN signature at baseline compared with non-responders. These results may suggest that monocyte frequency and STAT1 phosphorylation in circulating monocytes could represent early markers of response to baricitinib therapy.

KEYWORDS

interferon, STAT, JAKi, monocytes, rheumatoid arthritis, ISGs

Introduction

Rheumatoid arthritis (RA) is a systemic, chronic, autoimmune disease affecting the synovial joints as well as different organs (1). The activation of the innate and adaptive immunity (monocytes, macrophages, mast cells, neutrophils, dendritic cells, T lymphocytes, and B lymphocytes) is responsible for the inflammation of the synovial membrane, which in response determines a switch of synovial cells into fibroblast-like synoviocytes (FLS), responsible for the aggressive inflammatory phenotype of rheumatoid synovitis (1). Monocytes, macrophages, and dendritic cells are mononuclear phagocytes, and their roles in autoimmune and chronic inflammatory diseases, such as RA, are crucial (2, 3). The classification of monocyte subpopulations is based on the expression of the surface markers CD14 and CD16: the classical monocyte population (CD14+ CD16−/low), the intermediate monocyte population (CD14+CD16+), and the nonclassical monocyte population (CD14−/lowCD16+). The proportion of monocytes belonging to the intermediate and nonclassical groups may increase under different pathological conditions and may indicate RA disease activity and response to therapy (4). A previous analysis by Chara et al. evaluated the association between circulating monocyte number and subsets in RA patients treated with adalimumab, showing their predictive value for clinical response after six-month treatment (5).

The Janus-kinase (JAK) family includes JAK1, JAK2, JAK3, and TYK2 receptor-associated tyrosine kinases, able to activate the STAT (STAT1, 2, 3, 4, 5A, 5B, and 6) transcription factors. The JAK-STAT pathway is utilized by several type I and II cytokines, such as interferon (IFN), GM-CSF, and IL-6, that play a pathogenic role in RA (6). JAK-inhibitors are synthetic targeted molecules designed to inhibit the JAK activation pathway. Among the available JAK inhibitors, baricitinib preferentially acts on JAK1/JAK2.

The JAK-STAT pathway is one of the most important pathways for FLS proliferation and cartilage erosion through the secretion of matrix metalloproteinases (MMPs) (7). Few data are available on the effect of JAK inhibitors on FLS: IFNγ-induced adhesion and invasion ability are reduced (8), cell death is accelerated and thickening of the synovium is abrogated (9). Moreover, JAK-STAT is one of the intracellular signaling cascades used in the activation and differentiation process of monocytes; its inhibition with JAKi (tofacitinib and baricitinib) was associated with a surface phenotype similar to non-activated macrophages (Mφ) (10). Moreover, recent evidence seems to suggest that tofacitinib (JAK1/3 and, to a lesser extent, JAK2 inhibitor) can reduce not only the expression of inflammatory but also of fibrotic markers, also inhibiting the differentiation of macrophages toward a profibrotic phenotype (11). Additionally, the migration and mobility capacities of macrophages are also co-linked to JAK-STAT activity, as demonstrated by the altered chemotactic capacity of monocytes after peficitinib treatment (12).

This study verified the effects of baricitinib treatment on STAT phosphorylation in peripheral blood mononuclear cells (PBMCs) of RA patients and evaluated any correlation between STAT phosphorylation status and treatment response.

Materials and methods

Patients: inclusion criteria and study design

We enrolled consecutive patients referred to the Rheumatology outpatient clinic of the Sapienza University of Rome (Sapienza Arthritis Center) with a diagnosis of Rheumatoid Arthritis (RA) according to the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 criteria (13) from January to December 2018. All patients started baricitinib at 4 mg daily for RA that was moderately to severely active and with an inadequate response or intolerance to ≥1 conventional synthetic Disease Modifying Antirheumatic Drugs (csDMARDs), as for local indications. All patients signed a dedicated informed consent for participation in this study. Before starting baricitinib, all patients were screened for latent tuberculosis, previous hepatitis B/C virus infections, and varicella-zoster. Patients with ongoing or recent infections, a history of malignancy in the past 5 years, or any other condition that contraindicated the initiation of baricitinib were excluded from the study. Baseline demographic data (ethnicity, sex, age) and clinical data were collected: weight, height, body mass index (BMI), duration of disease, positivity for rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA), number of previous csDMARDs, and biological Disease Modifying Antirheumatic Drugs (bDMARDs). The disease activity was assessed at the beginning and then after 4–12–24 weeks of therapy (T0–T1–T3–T6), by the Disease Activity Score 28 (DAS28) calculated using C-Reactive Protein. Additionally, the assessment of disease activity and pain (PhGA) and the patient global assessment (PGA) by the physician were measured using a visual analog scale (VAS 0–100 mm). Patients were considered responders if, after 4 weeks, they had achieved at least a moderate EULAR response (14). For this study, we selected seven consecutive responders and seven consecutive non-responder patients.

Preparation of human peripheral blood mononuclear cells

Patient blood samples were collected on the same day of treatment. The whole blood was first diluted in PBS 1× at a 1:1

ratio, then gently layered on a volume (equivalent to the starting blood volume) of Lympholyte (Cedarlane) and centrifuged at 870g at room temperature for 25 min with low acceleration/brake. PBMCs were collected at the interface between the layers of Lympholyte and diluted blood and washed with PBS 1×. After cell counting, PBMCs were frozen in cryovials in FBS 10% DMSO and kept at −80°C until the experiment day. PBMCs of all patients and at all time points were longitudinally collected and frozen, to be thawed together on the day of the experiment.

Flow cytometry for pSTATs

On the day of the analysis, PBMCs were thawed at 37°C, counted and seeded in a 96-well plate. Cells were labeled with viability dye for 30 min at room temperature, then with CD127 Alexa Fluor 647 (since this epitope was sensitive to subsequent fixation), followed by cytokine stimulation that was performed for 15 min at 37°C with a cocktail of IL-6 100 ng/ml (PeproTech), IL-2 100 ng/ml (Roche), and IFN-α2b 40.000 IU/ml (Miltenyi Biotec). Then, cells were fixed and permeabilized using Cytofix Fixation Buffer (BD Biosciences) pre-warmed to 37°C and Perm Buffer III (BD Biosciences) pre-cooled to −20°C, according to the instructions of the manufacturer. Finally, intracellular staining was performed with a cocktail of fluorochrome-conjugated antibodies in PBS 0.5% BSA for 30 min at room temperature. The staining was performed according to the following panel: Viability Dye eFluor780 (65-0865-14, Invitrogen), CD127 Alexa Fluor 647 (558598, BD Biosciences), CD4 PerCP-Cy 5.5 (560650, BD Biosciences), CD8 Brilliant Violet 510 (563919, BD Biosciences), CD14 Brilliant Violet 605 (564054, BD Biosciences), FOXP3 Alexa Fluor 700 (56-4776-41, Invitrogen), CD45RA Brilliant Violet 785 (304140, Biolegend), phospho-STAT1 Alexa Fluor 488 (612596, BD Biosciences), phospho-STAT3 Pacific Blue (560312, BD Biosciences), phospho-STAT4 PE (558249, BD Biosciences), and phospho-STAT5 PE-Cy7 (560117, BD Biosciences). In all flow cytometry experiments, cells were acquired on LSR Fortessa (BD Biosciences).

Flow cytometry for pSTAT1 in monocytes

Thawed cells were labeled with viability dye in PBS for 20 min at 37°C, followed by surface staining for 15 min at room temperature in PBS 2% FBS. After stimulation with IFN-α2b 40.000 IU/ml (Miltenyi Biotec) for 15 min at 37°C, cells were fixed and permeabilized using Cytofix Fixation Buffer (BD Biosciences) pre-warmed to 37°C and Perm Buffer III (BD Biosciences) pre-cooled to −20°C, according to the

instructions of the manufacturer. Fc Block (Human TruStain FcX, Biolegend) in PBS was used to prevent the unwanted binding of Fc receptor CD16 antibody. Finally, intracellular staining was performed for 30 min at room temperature in PBS with 0.5% BSA. The following panel was used: Viability Dye Aqua Brilliant Violet 510 (L34957 A+B, Invitrogen), CD3 APC-Cy7 (317341, Biolegend), CD19 APC-eFluor780 (47-0199-42, Invitrogen), CD56 APC-eFluor780 (47-0567-42, eBiosciences), CD14 BB700 (566466, BD Biosciences), CD16 Alexa Fluor 647 (557710, BD Biosciences), HLA-DR PE-CF594 (562304, BD Biosciences), and phospho-STAT1 Brilliant Violet 421 (566238, BD Biosciences).

Intracellular staining of interferon stimulated genes

For the analysis of the protein levels of selected ISGs (ISG15, PKR, and MX1), PBMCs were thawed at 37°C, counted, and seeded in a 96-well plate, and then analyzed directly or stimulated overnight at 37°C with or without IFN-α2b 40.000 IU/ml (Miltenyi Biotec). After stimulation, cells were labeled with a viability dye for 30 min at room temperature in PBS, followed by surface staining in PBS 2% FBS for 20 min at 4°C. Cells were then fixed and permeabilized using the FOXP3/Transcription Factor Fixation/Permeabilization Buffers (eBioscience) according to the instructions of the manufacturer. Fc Block (Human TruStain FcX, Biolegend) in PBS was used to prevent the unwanted binding of Fc receptor CD16 antibody. Finally, intracellular staining was performed for 30 min at room temperature in Permeabilization Buffer (eBioscience). The flow cytometry panel was the following: Viability Dye eFluor780 (65-0865-14, Invitrogen), CD14 Brilliant Violet 421 (563743, BD Biosciences), MXA Alexa Fluor 488 (AMab237298, Abcam), PKR Alexa Fluor 647 (AMab224921, Abcam), and ISG15 PE (IC8044P, R&D Systems).

Statistical analysis

Analysis was performed using Flowjo 10.8 and Prism 8.0 (GraphPad Software) for statistical tests and graphical presentations. Data are presented as means ± SD. Two-way ANOVA with Geisser–Greenhouse correction and Sidak's multiple comparisons test was used to assess intergroup differences in longitudinal samples. A Mann–Whitney test was used to assess differences between subgroups. All the P-values lower than 0.1 are shown. A P-value of <0.05 was considered statistically significant.

Results

Dynamics and phosphorylation of STATs in selected immune cell subtypes in responder and non responder patients

Investigating a possible immunological marker of clinical response to baricitinib in RA patients, we longitudinally analyzed the frequencies and the STAT phosphorylation status of four selected immune cell types, i.e., monocytes, CD4 conventional T cells (Tconv), regulatory T cells (Treg), and CD8 T cells, identified according to the gating strategy shown in Supplementary Figure 1A. The main characteristics of responder (R = 7) and non-responder (NR = 7) patients are summarized in Table 1.

At baseline, the frequency of monocytes and CD8 T cells tended to be lower in R patients than in NR patients (n = 4/group); the percentage of monocytes remained lower at all the time points, reaching statistical significance at T3; conversely, Tconv and Treg percentages were similar in R and NR and were not affected by treatment with baricitinib (Supplementary Figure 2A).

Then, we assessed the phosphorylation status of multiple STATs (1, 3, 4, and 5) in these immune cell subsets, either unstimulated or briefly exposed to a cytokine cocktail pulse (IL-2, IL-6, and IFNα), to estimate the pre-existing STAT activation, and the responsiveness to a *de novo* stimulation, respectively. Overall, the stimulation increased the phosphorylation of STAT 1, 3, and 5 in all cell subpopulations (while pSTAT4 was only detected at low levels in Tconv and CD8 T cells) (Supplementary Figure 3), as also evident in a multidimensionality reduction plot (Supplementary Figure 4). When we analyzed the single STAT profiles, we did not find significant differences between R and NR patients at any time point, STAT, or cell type; however, we observed a trend, especially regarding monocytes (Supplementary Figure 2B). Indeed, basal STAT1 phosphorylation tended to be lower in monocytes of R patients at all time points; conversely, cytokine-induced STAT1

phosphorylation was slightly reduced from T0 to T1 (but not at T3) in R but not in NR patients (Supplementary Figure 2B, upper left panel).

Based on this preliminary screening, we thus focused our analysis on STAT1 phosphorylation at early time points in monocytes, which were identified using a more conservative gating strategy (Supplementary Figure 1B). This analysis highlighted that, at T0, samples of R patients displayed a trend towards lower monocyte percentage and higher cytokine-induced STAT1 phosphorylation. Both monocyte percentage and cytokine-induced STAT1 phosphorylation decreased from T0 to T1 only in R and not in NR patients (Figure 1). These results suggest that monocyte dynamics and cytokine responsiveness in terms of STAT1 phosphorylation might represent an immune target associated with the clinical response to baricitinib.

Monocyte frequency and STAT1 phosphorylation after IFNα stimulation decreased in responder patients

Recent data showed the influence of increased levels of serum IFNα in predicting the response to TNFα inhibitors in RA patients (15); considering the effect of the cytokine cocktail stimulation on STAT1 phosphorylation in R versus NR patients, we decided to investigate and isolate the effect of IFNα. We therefore focused the subsequent analyses on the characterization of monocyte response to IFNα, in PBMCs collected at T0, T1, and T3 from R and NR patients (n = 5–6/group) (Table 1). For this analysis, monocytes were gated using a stringent strategy as CD3− CD56− CD19− HLADR+ CD14+/− CD16+/− live single cells (Supplementary Figure 5). When monocyte frequency was analyzed using this strategy, we confirmed that R patients tended to have a lower monocyte frequency at T0 compared with NR, and that only R patients experienced a decrease in monocyte frequency from T0 to T1,

TABLE 1 Clinical and demographic features of enrolled RA patients.

Clinical and demographic features of enrolled patients	Responders n = 7	Non-responders n = 7	*p*
Age (years), mean ± SD	52.77 ± 13.5	59.95 ± 13.18	ns
Female : Male	7:0	5:2	ns
BMI*, mean ± SD	24.03 ± 1.53	24.53 ± 2.38	ns
Disease duration (months), mean ± SD	137 ± 73.6	228 ± 136.12	ns
Rheumatoid Factor, n (%)	4 (57.14)	3 (42.86)	ns
ACPA, n (%)	4 (57.14)	3 (42.86)	ns
DAS28CRP at baseline visit, mean ± SD	5.53 ± 1.4	5.82 ± 1.17	ns
Number of previous bDMARDs, n (%)	3.14 ± 1.86	3.43 ± 1.9	ns
Baricitinib in monoterapy, n (%)	5 (71.42)	3 (42.86)	ns
Daily PDN dose, mean ± SD	2.28 ± 3.86	4 ± 3.62	ns

SD, Standard Deviation; BMI, Body Mass Index; ACPA, anti-citrullinated peptide antibodies; DAS28CRP, Disease Activity Score 28 calculated with C-Reactive Protein; bDMARDs, biological Disease Modifying Antirheumatic Drugs; PDN, prednisone.
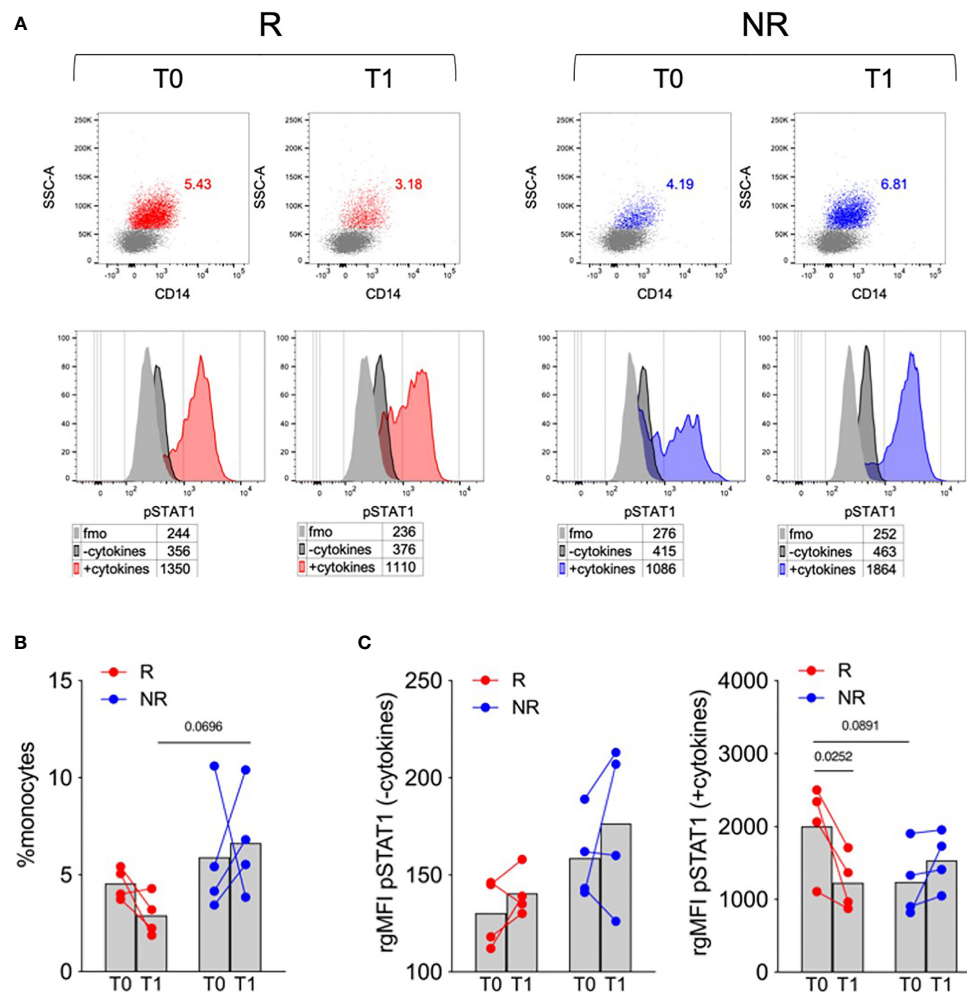
FIGURE 1

Monocyte frequency and STAT1 phosphorylation in response to a cytokine cocktail in R versus NR patients. **(A)** Representative overlays showing monocytes percentages in PBMCs (upper panels) and STAT1 phosphorylation (pSTAT1) in untreated (−cytokines) or cytokine cocktail-stimulated (+cytokines) monocytes (lower panels) in PBMCs of R and NR patients (n = 4/group), collected before (T0) and after 1 (T1) month post baricitinib therapy starting. Numbers in tables indicate the gMFI of pSTAT1 in each condition; fmo, fluorescence-minus-one control. **(B, C)** Cumulative analysis of monocyte percentage **(B)** and the rgMFI of pSTAT1 in monocytes **(C)**, calculated by subtracting the gMFI of the respective fmo control, in PBMCs of R and NR patients (n = 4/group), collected before (T0) and after 1 (T1) month post baricitinib therapy starting. Bars represent means *P*-values were calculated by 2-way ANOVA with Geisser−Greenhouse correction and Sidak's multiple comparisons test.

partially restored at T3 (Figures 2A, B). The three subpopulations of classical (CD14+CD16−), intermediate (CD14+CD16+), and nonclassical (CD14−CD16+) monocytes were identified: at all the tested time points, classical monocytes represented most monocytes, and their frequency declined from T0 to T1 in R patients (Figures 2C, D).

We then analyzed the percentage of classical monocytes, either unstimulated or shortly pulsed with IFNα, that were positive for phosphorylated STAT1 (Figure 3A). Unstimulated cells showed a certain proportion of phosphorylated STAT1 that decreased only in R patients from T0 to T1 and significantly to T3 (Figure 3B). After IFNα stimulation, we

did not find any significant difference in the percentage of pSTAT1+ cells in R compared with NR patients; however, there was a trend for higher pSTAT1+ monocytes at T0, which was reduced at T3, in the monocytes of R patients compared with NR (Figure 3C). Consequently, the difference in pSTAT1 + cell percentage between unstimulated and stimulated cells tended to increase from T0 to T1 and T3 in R but not in NR patients (Figure 3D). These data support the idea that the modulation of basal STAT1 phosphorylation in monocytes is associated with the clinical response to baricitinib and that therapy may target the response of monocytes to cytokines besides IFNα.
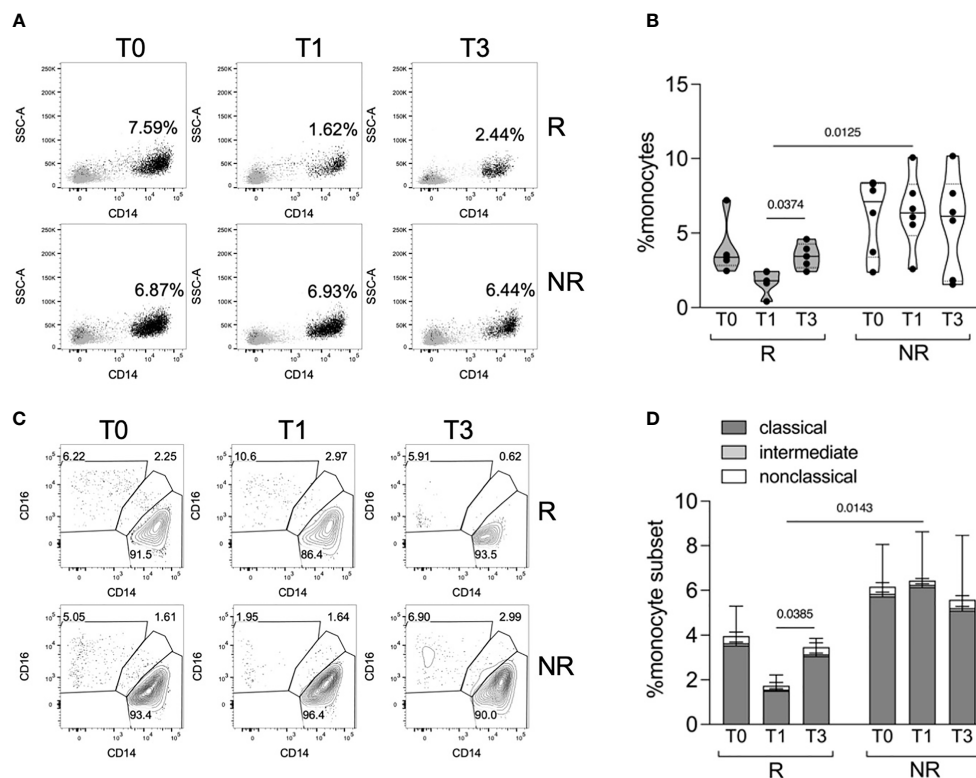
FIGURE 2

Monocyte frequency and subpopulations in R versus NR patients. **(A, B)** Representative overlays **(A)** and distributions **(B)** of monocyte percentages in PBMCs of R (n = 5) and NR (n = 6) patients, collected before (T0) and after 1 (T1) and 3 (T3) months post baricitinib therapy starting. *P*-values were calculated by 2-way ANOVA with Geisser–Greenhouse correction and Sidak's multiple comparisons test. **(C, D)** Representative contour plots **(C)** and stacked histograms **(D)** showing the percentage of classical, intermediate, and nonclassical monocytes in PBMCs of R (n = 5) and NR (n = 6) patients at the indicated time points. *P*-values were calculated by 2-way ANOVA with Geisser–Greenhouse correction and Sidak's multiple comparisons test, comparing classical monocyte frequencies in the indicated conditions.

Monocytes from responder patients basally express PKR, but not ISG15, at higher levels compared to non-responder patients

The data collected so far indicate that R patients had higher STAT1 phosphorylation both basally and in response to IFNα stimulation. IFNα responsiveness can be modulated by the pre-existing IFN signature. We analyzed, through intracellular flow cytometry, the expression of ISG15—an IFNα-negative regulator (16), with two other two ISGs, namely, PKR and MXA, which do not play any known inhibitory role on STAT1. The expression of these genes was analyzed *ex vivo* or after a culture overnight with IFNα, as a positive control. All three proteins were upregulated after *in vitro* culture with IFNα, in monocytes from both R and NR patients (Figure 4). ISG15 was slightly higher in monocytes from R patients compared to NR *ex vivo*, but the difference was no more evident after culture, irrespective of IFNα addition (Figure 4B). Conversely, PKR expression was significantly higher in monocytes from R

patients both *ex vivo* and after culture and tended to be higher in R than in NR after IFNα stimulation (Figure 4C). Finally, MXA expression tended to be higher in R compared to NR only at baseline (Figure 4D). These results confirm the hypothesis that R patients may have a pre-existing IFN-STAT1 signature in their monocytes, which, however, does not prevent subsequent IFN responses, and which may be a target of baricitinib therapy.

Discussion

The inhibition of the JAK-STAT pathway in RA treatment has gained increasing interest given the pleiotropic effects of this class of drugs in immune and non-immune cells (16). In this pilot investigation, we focused on the role of JAK-inhibition with baricitinib in circulating monocytes. Our results suggest that monocyte frequency and the IFN signature in circulating monocytes represent early markers of response to baricitinib in RA patients. Indeed, we observed that a higher baseline
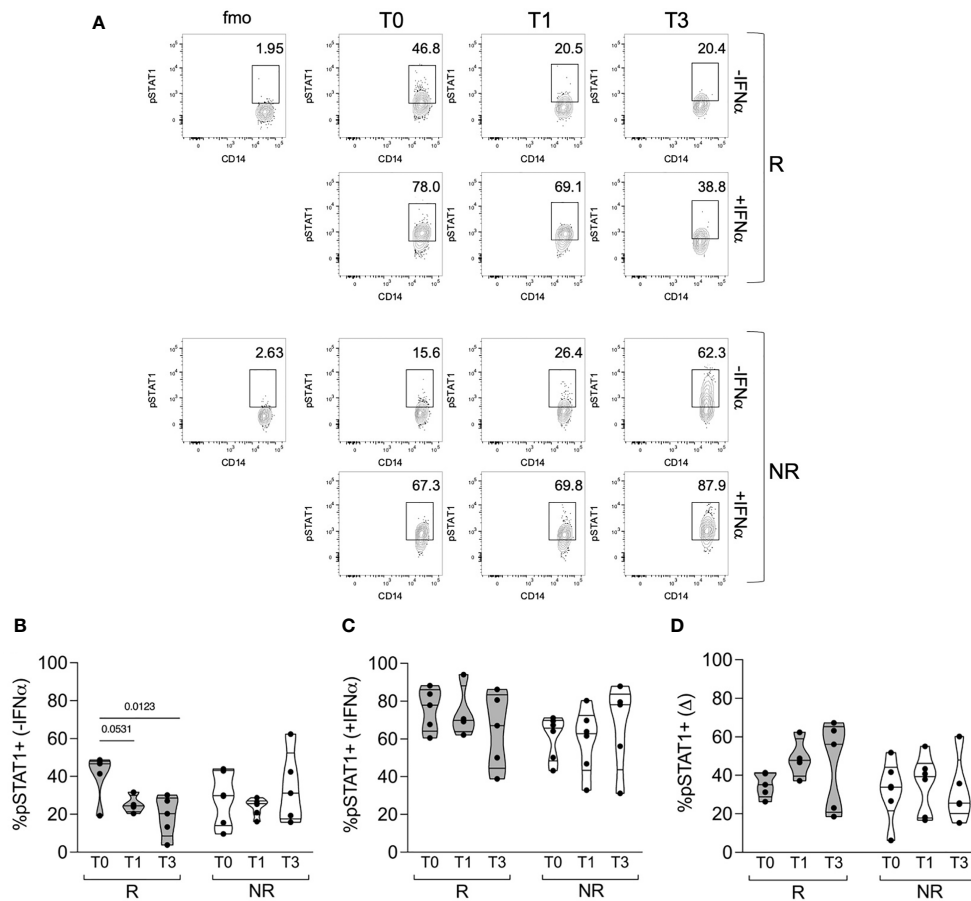
FIGURE 3

STAT1 phosphorylation in monocytes of R versus NR patients. **(A)** Representative contour plots showing the percentages of pSTAT1+ cells in gated classical monocytes from PBMCs of one R and one NR patient, collected before (T0) and after 1 (T1) and 3 (T3) months post baricitinib therapy starting. Cells were stained after culture for 30 min with (+IFNα) or without (−IFNα) $4 * 10^4$ IU/ml of IFNα. **(B−D)** Cumulative distributions of the percentages of pSTAT1+ cells without IFNα stimulation **(B)** or with IFNα stimulation **(C)**, and of the difference (Δ) between the two values calculated for each sample and condition **(D)**. The analysis was performed in PBMCs of R (n = 5) and NR (n = 6) patients at the indicated time points. Samples were included in the analysis only if enough cells were found in the monocyte gate. P-values were calculated by 2-way ANOVA with Geisser−Greenhouse correction and Sidak's multiple comparisons test.

expression of IFN-related genes and STAT1 phosphorylation characterizes patients who will respond to baricitinib.

The first result of the study highlighted a reduction in the frequency of circulating monocytes after 4 weeks of baricitinib therapy only in responder patients; thus, the early reduction in the percentage of circulating monocytes could be able to discriminate responder patients.

Patients with RA have significantly higher percentages of CD14+CD16+ monocytes than healthy subjects (17). Previous studies have shown that glucocorticoids and TNF-inhibitors can reduce CD14+CD16+ monocytes both *in vivo* and *in vitro* (5, 18). A research aimed at studying the effect of TNF-inhibitors on the percentage of monocytes and their ability to differentiate into osteoclasts showed that these drugs could decrease the frequency of classical monocytes (CD14bright/CD16−) after about 6 months of treatment, possibly

contributing to the decrease of erosive damage during therapy with TNF-inhibitors (19). Our data suggested that baricitinib could also have the same effects on classical monocytes: the change in CD14+/CD16− cells mostly accounted for the decrease in the percentage of monocytes detected in responder patients compared with non-responders. In another study, TNF-inhibitors significantly decreased CD14hi/CD16− monocytes compared with placebo, whereas the CD14dim/CD16+ monocyte subclass increased significantly; the authors concluded that the shift toward nonclassical monocytes (CD14dim/CD16+) might reflect the recruitment of this cell type to the inflammatory site, an event associated with reduced disease activity (20). Indeed, CD16bright/CD14+ monocytes showed a correlation with disease activity in RA, as evaluated by clinical indices and ultrasound (21, 22).

FIGURE 4

Expression of ISGs in monocytes at therapy starting of R versus NR patients. **(A)** Representative histogram overlays plots showing the expression of ISG15, PKR, and MXA by intracellular staining in gated classical monocytes from the PBMCs of one R and one NR patient collected at baricitinib therapy starting (T0). Gray histograms represent the fmo, fluorescence-minus-one control; red represents cells stained *ex vivo*; green represents cells stained after overnight (o.n.) culture; blue represents cells stained after o.n. culture in the presence of $4 * 10^4$ IU/ml of IFNα. Numbers in tables indicate the gMFI. **(B–D)** Cumulative distributions of the intracellular expression (measured as the gMFI) of ISG15 **(B)**, PKR **(C)**, and MXA **(D)**, in gated classical monocytes from PBMCs of R and NR patients (n = 5/group) collected at baricitinib therapy starting (T0), and stained *ex vivo* or after overnight (o.n.) culture in the absence or the presence (o.n. + IFNα) of $4 * 10^4$ IU/ml of IFNα. The dotted lines indicate the average fmo values for each protein. *P*-values were calculated by Mann–Whitney test. .

We showed a trend in monocyte frequency before starting treatment with baricitinib, with lower levels in R patients compared with NR (not significant). We could not correlate this event with disease activity at baseline, since R and NR patients did not present significantly different disease activity at baseline (data not shown).

Type I IFNs are considered crucial in the activation and differentiation of monocytes, as well as in other innate immune cells, and patients with autoimmune conditions have a peculiar "IFN signature" (23, 24). This study confirmed the effect of baricitinib on STAT1 phosphorylation in circulating monocytes, leading us to hypothesize that STAT1 phosphorylation is influenced by a different monocyte priming in patients who will respond to baricitinib therapy than in those who will not respond. Among the tested cytokines, our data suggest that IFNα contributes to the basal phosphorylation status of STAT1 detected in patients who will respond to baricitinib. Indeed, STAT1 activation is involved in the signaling cascade of all IFNs.

Generally, binding of IFNs to their receptors leads to JAK1 and STAT1 engagement and conformational changes to the active form (25, 26).

Both infiltration of IFNγ-producing T lymphocytes (27) and elevated levels of STAT1 and STAT target genes (28) have been observed in rheumatoid synovium, and treatment with DMARDs was able to reduce synovial STAT1 expression only in patients in whom the treatment was effective (29). These data confirm the evidence that the IFN/JAK1-2/STAT1 signaling pathway is involved in rheumatoid synovial inflammation.

The IFN signature, i.e., the set of genes induced by IFN, plays a crucial role in RA pathogenesis, even from the earliest stages of the disease onset. In a study on patients with inflammatory arthralgia, naïve to any treatment, the authors demonstrated that patients who would develop RA had a specific IFN signature, with an up-regulation of SIGLEC1 and MS4A4A, differently from patients who presented with non-inflammatory arthralgia (30). Moreover, the authors showed that the genes MSA4A,

PDZK1IP1, and EPHB2, at baseline and follow-up, were able to discriminate patients with RA from healthy controls, assuming a predictive value for RA development (30). In established RA, IFN-related genes influence different cell functions such as apoptosis, gene transcription, protein degradation, Th2 induction, and B lymphocyte proliferation (31). Castañeda-Delgado et al. evaluated the different expression of IFN-related genes in different disease stages, correlating their levels with the production of ACPAs. They enrolled patients with RA in the early stages and in established disease, comparing them with patients at high risk of developing RA according to the positivity of ACPA. Those patients with definite diagnosis and/or long-standing disease showed an increased expression of ISG15 compared with early RA patients. In addition, the IFN signature was significantly correlated with the positivity for ACPA and anti-carbamylated protein antibodies (32).

ISG15 is a member of the ubiquitin family, involved in the regulation of various cellular activities, including protein stability, intracellular trafficking, cell cycle control, and immune modulation. ISG15 and downstream members of ISG15 activation are strongly induced by type I IFN and exert negative feedback on the IFN pathway (33). ISG15 protects Tconv cells from STAT1 phosphorylation and induces resistance to IFNα-mediated depletion in Tregs, exerting negative feedback, which can be explained by a desensitizing effect from IFN stimulation (16). Less is known about ISG15 and monocytes in RA. Our results showed that IFNα could induce ISG15 in the whole cohort without a significant difference between responders and non-responders.

To the best of our knowledge, no previous studies have evaluated the effects of JAK inhibitors on a pre-existing IFN signature and on IFN-induced modification of STAT1 phosphorylation in patients with RA. A phase 2 study with baricitinib in patients with systemic lupus erythematosus (SLE) showed a significantly higher expression of the STAT1 gene in SLE patients who had active disease at study entry. Additionally, significant overexpression of STAT1 coupled with STAT2 at baseline correlated with IFN predominance (34). In these patients, treatment with baricitinib reduced mRNA expression of the target genes of transcription factors STAT2 and STAT4, but not STAT1 (34). This analysis was restricted to the baseline IFN genes and did not mention the impact of baricitinib on STAT phosphorylation and IFN-related genes.

In our cohort, the greatest effect of baricitinib in terms of STAT phosphorylation was in STAT1, especially in monocytes.

Some literature data confirm different utilization of STATs in different immune cell subpopulations, supporting the role of STAT1 in monocytes (35). Previous work showed that circulating monocytes—and to a lesser extent, lymphocytes—from patients with RA exhibit higher levels of STAT1, correlating with disease activity (36).

In a recent work on patients with RA (n = 29) and Systemic Sclerosis (n = 21), the former showed a higher baseline level of STAT1 and STAT3 phosphorylation in CD3+ T cells and monocytes compared with healthy controls (37). The authors also investigated the effects of peficitinib, tofacitinib, and baricitinib on STAT phosphorylation induced by IL-2, IL-4, IL-6, IL-13, and IFN in T CD3+ lymphocytes and monocytes, showing that all JAKi were able to suppress STAT phosphorylation downstream of the different cytokines tested (37). Consistent with the results of the study by Kitanaga et al., our data confirmed that baricitinib exerts an inhibitory effect on STAT1 phosphorylation in monocytes stimulated with IL-2, IL-6, and, above all, IFNα.

In our work, STAT1 phosphorylation in monocytes differentiated responder and non-responder patients; after only 4 weeks, monocytes of responder patients were less responsive—in terms of STAT1 phosphorylation—to cytokine stimulation; in contrast, in non-responders, the extent of STAT1 phosphorylation was similar to that observed before therapy. We could therefore suggest that higher baseline STAT1 phosphorylation and the decrease in pSTAT1+ monocytes could be considered an early predictive marker of response to therapy. A previous study suggested an association between the phosphorylation of STAT6 and STAT1 in circulating leukocytes and the response to RA treatment (38). Kuuliala et al. analyzed the intracellular phosphorylation of STAT1 and STAT6 in response to IFNγ and IL-4 in RA patients treated with csDMARDs or bDMARDs (38). At baseline, in patients with treatment-naive RA, IFNγ-induced lymphocyte pSTAT1 and IL-4-induced monocyte pSTAT6 levels were higher in patients who achieved a good response to therapy than in non-responders to bDMARDS (38).

Monocytes are the peripheral blood counterparts of tissue synovial macrophages, the principal immune cell type infiltrating the inflamed synovia. In the tissue, FLS act as effectors, as they are responsive to the inflammatory local milieu, principally by TNF and IFN, transforming into pro-inflammatory behavior. *In vitro* studies have demonstrated that two JAK inhibitors, peficitinib and tofacitinib, suppress the FLS expression of apoptosis-resistant genes, inhibit the production of matrix metalloproteinases, and promote FLS death (9, 39). The T and B cells, macrophages and mast cells infiltrating the synovial tissue are possibly affected by the inhibition of the JAK-STAT pathway too. There is a crucial crosstalk between FLS and immune cells; *in vitro*, JAK inhibitors (tofacitinib, baricitinib, and upadacitinib) can suppress the secretion of different cytokines produced by FLS-stimulated T helper cells, as well as suppress T-cell proliferation, disrupting the circuits in the bidirectional interplay between FLS and immune cells (40). Tofacitinib and ruxolitinib suppress STAT1 activation in TNF-stimulated RA synovial macrophages (41). Moreover, tofacitinib can inhibit the expression of MMPs and interferon-regulated genes, in accordance with the reduction of pSTAT1 and pSTAT3 phosphorylation, supporting the role of IFNγ and IL-6 inhibition (42).

The results of this pilot study suggest that, with a decrease in monocyte frequency, the baseline cytokine (mostly IFNα)-induced phosphorylation of STAT1 could represent an early predictor of treatment response in RA patients starting baricitinib.

Data availability statement

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

Ethics statement

This study was reviewed and approved by the Ethical Committee of Policlinico Umberto I—Sapienza University of Rome. The patients/participants provided their written informed consent to participate in this study.

Author contributions

GT, CG, FS, and SP conceived the project, designed the protocol, wrote the draft, and analyzed the data. IP, MZ, APG, and FCe contributed to study design and monitored experimental procedure and analyzed the data. FCo, FCe, FS, and SP wrote the statistical analysis plan and analyzed the data, supervised the draft and revised the paper. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

CG, FCo and FS received speaker fees from Eli Lilly.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.932240/full#supplementary-material

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An overview of JAK/STAT pathways and JAK inhibition in alopecia areata

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Alopecia Areata (AA) is a common autoimmune disease characterized by non-scarring hair loss ranging from patches on the scalp to complete hair loss involving the entire body. Disease onset is hypothesized to follow the collapse of immune privilege of the hair follicle, which results in an increase in self-peptide/MHC expression along the follicular epithelium. Hair loss is associated with infiltration of the hair follicle with putatively self-reactive T cells. This process is thought to skew the hair follicle microenvironment away from a typically homeostatic immune state towards one of active inflammation. This imbalance is mediated in part by the dominating presence of specific cytokines. While interferon-γ (IFNγ) has been identified as the key player in AA pathogenesis, many other cytokines have also been shown to play pivotal roles. Mechanistic studies in animal models have highlighted the contribution of common gamma chain (γ_c) cytokines such as IL-2, IL-7, and IL-15 in augmenting disease. IFNγ and γ_c cytokines signal through pathways involving receptor activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). Based on these findings, JAK/STAT pathways have been targeted for the purposes of therapeutic intervention in the clinical setting. Case reports and series have described use of small molecule JAK inhibitors leading to hair regrowth among AA patients. Furthermore, emerging clinical trial results show great promise and position JAK inhibitors as a treatment strategy for patients with severe or recalcitrant disease. Demonstrated efficacy from large-scale clinical trials of the JAK inhibitor baricitinib led to the first-in-disease FDA-approved treatment for AA in June of 2022. This review aims to highlight the JAK/STAT signaling pathways of various cytokines involved in AA and how targeting those pathways may impact disease outcomes in both laboratory and clinical settings.

KEYWORDS

alopecia areata, JAK/STAT, JAK inhibition, cytokines, clinical trials

Introduction

Alopecia Areata (AA) is a prevalent autoimmune disease that carries a lifetime risk of approximately 2% (1). AA is characterized by nonscarring hair loss that typically appears as small patches on the scalp. Severe cases of AA can result in complete hair loss of the scalp (alopecia totalis, AT) or complete loss of hair on the entire body (alopecia universalis, AU). While this disease can affect people of all ages, the mean age range of the first instance of hair loss is 25 to 37 years of age (1). The physical loss of hair in AA patients is often accompanied with increased psychological burden, which may manifest as depression, anxiety, and/or an impaired quality of life when compared to healthy individuals (2). The cause of AA is obscure but is believed to arise from a combination of environmental influences and genetic factors, such as variants within loci or certain genes implicated in autoimmunity and T cell signaling (3). There is no cure for AA, and off-label treatments, such as topical or locally-injected agents or systemic immunosuppressants, are commonly used, although historically these agents have been shown to have variable or poor efficacy. In the past decade, a new strategy has emerged for treating AA which acts to dampen ongoing inflammation by inhibition of Janus kinase (JAK) signaling. As of June 2022, the FDA has approved one JAK inhibitor for the treatment of AA, marking the first and currently only on-label treatment for adults with AA. JAKs are signaling mediators that function to convert the engagement of cytokine receptors with cognate cytokine to downstream effects. Many cytokine pathways that contribute to the pathogenesis of AA, such as interferon-γ (IFNγ) and those in the common gamma chain (γ_c) family of cytokines, act by way of JAK signaling. Counteracting the effects of these cytokines with JAK inhibitors (JAKis) has shown efficacy in treating AA.

Relevant pathophysiology of AA

AA is a disease that affects the hair follicles, which are known to follow a cyclic physiologic pattern. Broadly, there are three phases: anagen, a growth phase; catagen, a transition phase; and telogen, a rest phase. The anagen hair follicle is known to reside in a state of immune privilege (IP), which is characterized by low levels of MHC protein expression in its inferior portion, a relative lack of danger or stress ligands, the presence of anti-inflammatory cytokines/hormones (TGFβ, IL-10, α-MSH) and a paucity of immune cell infiltrates (4, 5). This IP state is thought to help protect the follicle from unwanted immune-mediated attack during this time. Maintaining this evasive state may be particularly important during the anagen phase, where a large number of tissue-specific peptides and antigens are generated. However, the microenvironment of the hair follicle exhibits

dramatic changes in the AA disease state. In hair follicles in AA lesions, there is an increase in expression of MHC Class I and MHC Class II, upregulation of danger ligands (such as NKG2D activating ligands MICA and ULBP molecules), increased levels of proinflammatory cytokines, and a robust immune cell infiltrate (4, 6) (Figure 1). Immune cell infiltrates are comprised predominantly of CD4$^+$ and CD8$^+$ T cells. CD8$^+$ T cells in close association with the hair follicle were found to express NKG2D, an activating receptor commonly associated with the natural killer (NK) cell lineage (6). This population of CD8$^+$ NKG2D$^+$ T cells was found to be sufficient for induction of disease in the cell-transfer murine model of AA (7). Activated CD8$^+$ T cells are potent producers of pro-inflammatory cytokines, such as IFNγ, which skew the hair follicle microenvironment towards a proinflammatory state. Additionally, CD8$^+$ T cell cytotoxic activity is known to be enhanced in the presence of cytokines such as those of the γ_c family. CD4$^+$ T cells are also capable of producing cytokines that may contribute to the inflammatory state of the hair follicle during AA. Activated CD4$^+$ T cells are known to produce the γ_c cytokine IL-2, which may enhance CD8$^+$ T cell function. Additionally, a subset of CD4$^+$ T cells known as T helper type 1 (Th1) cells produce IFNγ in an activated state, which may contribute to the inflammatory setting of the AA hair follicle. Substantial evidence demonstrates that a variety of potent cytokines and their respective signaling pathways are contributing to the pathogenesis of AA by driving functional and phenotypic changes in populations of T cells and in the hair follicle.

FIGURE 1

The collapse of immune privilege in the anagen hair follicle during alopecia areata.
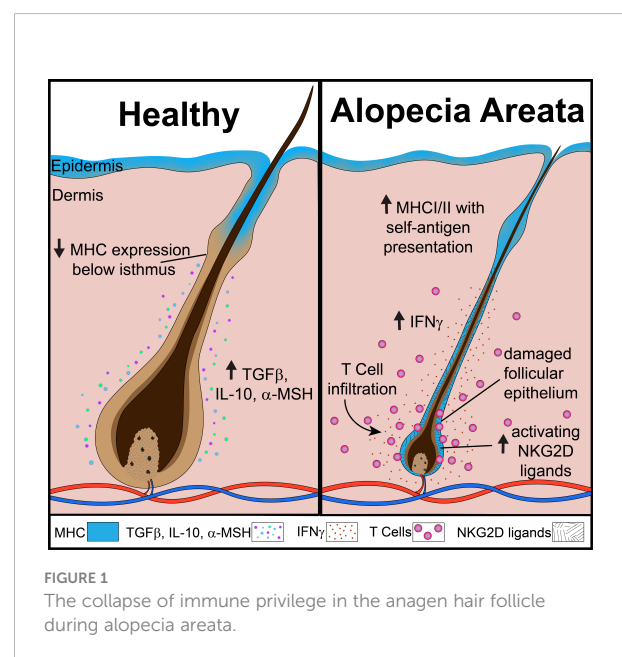
JAK/STAT signaling

A large number of cytokine receptors lack intrinsic kinase activity, necessitating intermediaries, including tyrosine kinases, to transmit their downstream signals. By engaging their receptors, many types of growth factors and cytokines elicit their effects through use of the JAK molecules (Figure 2). There are four members of the JAK family of tyrosine kinases: JAK1, JAK2, JAK3, and TYK2. They are unique from other families of tyrosine kinases by virtue of their domain structure involving seven similar JAK Homology (JH) domains. All members contain a kinase domain (JH1) at their C-terminus region, and the phosphorylation of a conserved tyrosine residue within this region stimulates their catalytic activity (8). At their N-terminal region, JAKs contain a Band-4.1, ezrin, radixin, moesin (FERM) domain that mediates their interactions with cytokine receptors (9). Inactive JAKs may be found uncomplexed in the cytosol or pre-associated *via* their membrane-proximal cytoplasmic region of cytokine receptor subunits at their Box1 and Box2 motifs. The sequence of these two motifs determines with which cytokine receptor the JAK molecule will interact (10). Cytokine ligation results in conformational changes in the receptor that lead to dimerization of receptor subunits and in the juxtapositioning of the pre-associated JAKs or the recruitment of free JAKs to their docking sites. This proximity allows for the auto- or trans-phosphorylation of conserved tyrosine (Tyr) residues of the kinase domain and subsequent activation of the JAKs. The activated JAKs then go on to phosphorylate Tyr residues found on the distal intracellular region of the cytokine receptor, creating a docking site for the Src homology-2(SH2) domains of recruited signal transducers and activators of transcription (STATs).

There are seven different members of the STAT family, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. They share six similar domains related to their function. All STATs contain a centrally located DNA-binding domain, which allows for recognition of, and binding to, DNA sequences of target genes. This is followed by the SH2 domain, which can recognize tyrosine-phosphorylated (p-Tyr) sites on cytokine receptor subunits to facilitate STAT docking; each cytokine receptor subunit contains different STAT binding motifs, and the sequence surrounding the p-Tyr dictates which STAT will bind with highest affinity (10). At the C-termini of STATs are the transcription activation domain (TAD), which contains serine phosphorylation sites that direct transcriptional activity and contain binding sites for nuclear cofactors (11). Upon their recruitment to docking sites on cytokine receptors, STATs are phosphorylated by JAKs at a tyrosine residue located between the SH2 domain and TAD. This phosphorylation results in the SH2 domain of each STAT releasing from the receptor subunit and instead binding to the p-Tyr site on the other STAT monomer, resulting in the formation of a STAT dimer in active conformation with exposed DNA-binding sites. Dimers then translocate into the nucleus and bind to target genes within the DNA that possess STAT-binding sites, which ultimately results in inflammatory gene transcription, and the production of pro-inflammatory cytokines (10).
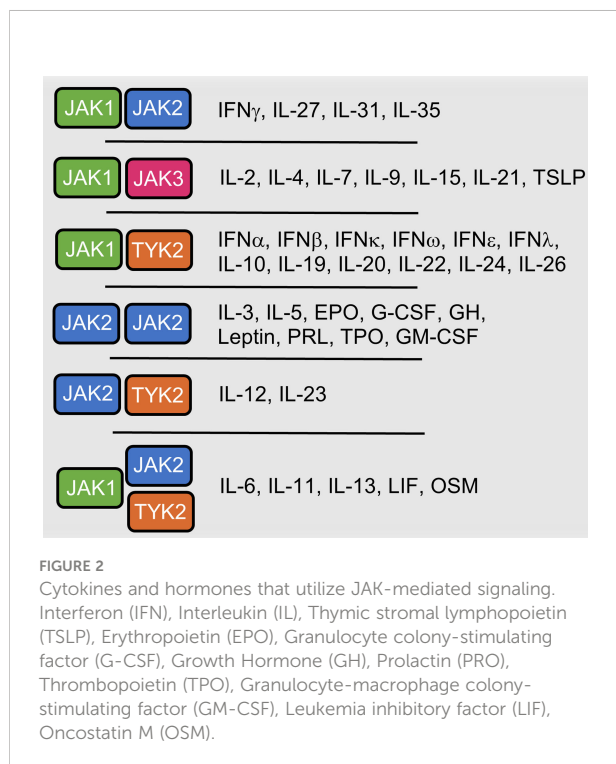
Cytokine signaling in AA

Pro-inflammatory cytokines have been shown to play key roles in the pathogenesis of AA. Mechanistic studies highlighting the importance of these cytokines in disease onset and development have been performed using murine models of alopecia. The C3H/HeJ (referred to C3H herein) strain of mice spontaneously develops hair loss that recapitulates many features of human AA. In particular, C3H AA is a non-scarring form of alopecia, exhibits waxing and waning cycles, and is marked by lymphocytic infiltrates around the hair follicle, including CD8^{+} and CD4^{+} T cells (12). Additionally, previously unaffected mice can be induced to develop AA in a reproducible manner by using two different methods. One method consists of grafting a piece of skin from an alopecic mouse, e.g. a mouse that spontaneously developed AA, onto the dorsal region of a naïve recipient mouse (13). Another method involves collecting the skin-draining lymph nodes from a previously-affected mouse, activating and expanding the T cell populations *in vitro*, and then injecting the resulting cell population intra-dermally into naïve recipient mice (14). The development of these induction methods, resulting in tractable murine AA models that rapidly

FIGURE 2

Cytokines and hormones that utilize JAK-mediated signaling. Interferon (IFN), Interleukin (IL), Thymic stromal lymphopoietin (TSLP), Erythropoietin (EPO), Granulocyte colony-stimulating factor (G-CSF), Growth Hormone (GH), Prolactin (PRO), Thrombopoietin (TPO), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Leukemia inhibitory factor (LIF), Oncostatin M (OSM).

develop disease in a reproducible manner, have made it feasible to study and unravel the pathogenesis of this disease and has furthered our knowledge obtained from human AA patients, which had been largely limited to observational or *in vitro* studies. In particular, the roles of IFNγ and γc family members, including IL-2, IL-7, and IL-15, have been specifically investigated. The findings from these studies will be discussed in more detail below.

Interferon-γ (IFNγ)

IFNγ is a 17 kDa protein that is biologically active as a homodimer; it is produced at high levels by activated CD4+ T helper type 1 (Th1) and CD8+ T cells, γδ T cells, and NK cells, and to a lesser extent by NKT cells, B cells, and antigen-presenting cells [15]. The secretion of IFNγ is often associated with host defense towards foreign pathogens and is known to aid in tumor surveillance and the anti-tumor response. IFNγ is the sole member of the Type II IFN class of cytokines and signals through a tetrameric receptor comprised of two IFNGR1 chains and two IFNGR2 chains. Nearly all types of mammalian cells express the IFNγ receptor and are thus responsive to the effects of IFNγ to some extent. The level of IFNγ sensitivity is largely controlled by the level of expression of the IFNGR2 chain [16]. Typically, IFNGR1 is readily expressed at relatively moderate levels, while the IFNGR2 chain can be upregulated from its low-level baseline expression during cases of activation or based on cell differentiation status [15]. IFNGR1 chains have binding sites for JAK1, and IFNGR2 chains have binding sites for JAK2. Cytokine recognition by the IFN-γ receptor leads to autophosphorylation of JAK2 proteins; these active JAK2 molecules then go onto trans-phosphorylate JAK1 proteins. Activated JAK1 molecules each phosphorylate a tyrosine residue on their respective IFNGR1 chain, leading to the dual recruitment and activation of STAT1 monomers [16]. Active STAT1 homodimers, also known as gamma-activated factors (GAFs), translocate to the nucleus and bind to the gamma-activated sequence (GAS) to drive target gene transcription [17].

In the setting of AA, sera from patients contain significantly higher levels of IFNγ when compared to healthy patients [18–20]. IFNγ is hypothesized to play an important role in the collapse of the immune privileged state of the hair follicle. The ability of IFNγ to cause IP collapse has been demonstrated in experiments where anagen-stage hair follicles collected from a healthy scalp were cultured in the presence of IFNγ, resulting in increased expression of MHC I molecules [21]. In a murine skin-graft induction model of AA, IFNγ has been shown to play a critical role in disease onset, as IFNγ-deficient mice fail to develop disease after grafting [22]. Mice treated with an IFNγ neutralizing antibody starting at the time of grafting were prevented from developing disease, had a reduction in skin-infiltrating CD8+ NKG2D+ T cells, and failed to upregulate

MHC I and MHC II molecules in the skin [7]. Interestingly, groups have shown that intradermal injection of IFNγ alone or in combination with poly[I:C] into anagen-induced skin of naïve mice can drive the emergence of AA [23, 24]. Overall, IFNγ has been identified as one of the main cytokines contributing to disease and does so, at least in part, by driving changes in the follicular epithelium to disrupt hair follicle immune privilege.

Interleukin-2 (IL-2)

IL-2 is a 15 kDa protein that is mainly produced by activated CD4+ T cells and, to a lesser extent, by activated CD8+ T cells and DCs, NK cells, and NKT cells [25]. IL-2 can signal through a high-affinity trimeric receptor (K_d ~10^{-11} M) or an intermediate-affinity dimeric receptor (K_d ~10^{-9} M) [26]. The trimeric receptor consists of a unique IL-2Rα (CD25) subunit, the shared IL-2/15Rβ (CD122) subunit, and the γc (CD132) subunit, while the dimeric receptor consists of only the β and γc subunits. In order to form the trimeric receptor, IL-2 first weakly binds to IL-2Rα (K_d ~10^{-8} M); this initial interaction causes a conformational change in IL-2 that allows for association with the β subunit, recruitment of the γc subunit, and leads to the formation of a stable IL-2/receptor complex [27]. Expression of the high-affinity, trimeric IL-2R is observed in CD4+ FoxP3+ regulatory T cells (Tregs) and activated conventional CD4+ or CD8+ T cells, suggesting that these populations of cells are exquisitely responsive to the effects of IL-2. The intermediate-affinity, dimeric IL-2R can be found on CD8+ memory T cells and NK cells, indicating they may also respond to IL-2. The dimeric receptor can also engage IL-15, and this promiscuity results in competition of the two cytokines for this receptor.

IL-2Rα is not known to engage any intracellular signaling molecules, whereas the IL-2Rβ subunit engages JAK1, and the γc subunit engages JAK3. Activation of JAK1/3 leads to subsequent activation and formation of STAT5/5 or STAT3/5 dimers that translocate to the nucleus to drive gene transcription programs that promote cellular expansion, survival, and maintenance. Additionally, STAT5 plays an important role in influencing T cell lineage fate. STAT5 is known to drive the expression of the Th1/Tc1 fate determining transcription factor T-bet which is crucial for acquisition of effector phenotype and functions, such as inducing CXCR3 expression and IFNγ production [28]. There is also a STAT5 binding site in the CNS2 region of the *FOXP3* gene, which implicates STAT5 as a stabilizer of Foxp3 expression, which is important for the potent immunosuppressive phenotype and function of Tregs [29]. Thus, IL-2 is a pleotropic cytokine that can enhance both effector and regulatory T cell populations that may work against each other in the context of an autoimmune disease such as AA.

In AA patients, increased mRNA expression of IL-2 is detected in the deep dermis of the skin, which contains the bulb

of the hair follicle, when compared to levels of the upper dermis (30). Furthermore, active lesions of AA patients contain higher amounts of IL-2 mRNA than inactive areas, suggesting that IL-2 may be contributing to the localized areas of disease. A GWAS of AA patients and healthy controls revealed disease associations with SNPs in both the IL-2 gene and the IL-2Rα (CD25) gene (31). Various groups have profiled the serum of AA patients and observed higher levels of IL-2 in circulation in comparison to healthy patients (19, 20, 32). The crucial role of IL-2 has also been demonstrated with the murine skin-graft induction model. Graft-mediated induction experiments with IL-2$^{+/-}$ mice, which possess only one functional IL-2 allele and produce 50% of typical IL-2 amounts, revealed that these mice were partially resistant to disease induction and had reduced leukocyte infiltration into skin lesions (33). Other reports demonstrated that mice treated with an anti-IL-2 neutralizing antibody at the time of grafting failed to develop disease, had a marked reduction of pathogenic CD8$^+$ NKG2D$^+$ T cells infiltrating the skin, and prevented the upregulation of MHC Class I and Class II in skin (7). Overall, IL-2 plays an essential function in T cell proliferation and homeostasis. The important roles of IL-2 in AA outlined above provides growing support for the role of γ$_c$ cytokine family members in this disease and its relevant animal models.

Interleukin-7 (IL-7)

IL-7 is a 17 kDa protein that is produced by various non-hematopoietic cells, such as stromal cells in the lymphoid organs or epithelial cells in the thymus or intestine (34). IL-7 signals through a dimeric receptor consisting of a unique IL-7Rα (CD127) subunit and the γ$_c$ subunit. Expression of the IL-7 receptor can be found on naïve and memory αβ T Cells and is crucial for their development, survival, proliferation, and overall homeostasis. The IL-7Rα subunit preferentially binds JAK1, while the γ$_c$ subunit binds JAK3. Activation of JAK1/3 leads to the recruitment and activation of STAT5, prompting the formation of a STAT5 homodimer that activates target genes (35). Genes transcribed after IL-7 signaling include the anti-apoptotic gene, *BCL2*, and genes that promote cell-cycle progression and differentiation, such as *MYC* and *PIM1* (36).

In AA patients, T cells infiltrating the HF were shown to have increased expression of IL-7Rα in comparison to T cells in control scalp samples (37). Furthermore, PBMCs isolated from AA patients stimulated in the presence of IL-7 resulted in significantly higher IFNγ production by CD8$^+$ T cells in comparison to control patients (37). GWAS data has revealed one SNP in the IL-7Rα region associated with AA patients (31, 37). Together this suggests that T cells in both the circulation and skin of AA patients are more responsive to IL-7, and this may enhance their effector phenotype that promotes ongoing disease in these patients.

Murine studies of alopecia have demonstrated similar findings regarding the role of IL-7 and have also begun to unravel mechanisms of action. Gene expression analysis has revealed higher levels of IL-7 in AA skin when compared to skin of unaffected (UA) mice (37). The increase in IL-7 is likely due to increased production by keratinocytes, as they are known to upregulate IL-7 expression in the presence of IFNγ, which is likely released by CD8$^+$ T lymphocytes infiltrating alopecic skin (37, 38). This suggests that a feedback loop exists between IL-7 production and IFNγ production of resident and infiltrating immune cells within the skin that may enhance the local pro-inflammatory environment. Mice treated with exogenous IL-7 complexed to an anti-IL-7 antibody, which expands the lifespan of IL-7 *in vivo*, experienced significantly quicker disease onset than mice receiving isotype control in a skin-graft induction setting. Additionally, mice treated with an anti-IL-7Rα blocking antibody at the time of skin-graft induction were completely resistant to development of disease. Clinical relevance of IL-7 blockade was demonstrated by the treatment of mice with anti-IL-7Rα after the first instance of hair loss. This treatment led to hair-regrowth in 60% of mice and reduced the inflammatory profiles of skin (37). While IL-7 is one of the more recent cytokines shown to contribute to the pathogenesis of AA, it again highlights the pertinent involvement of γ$_c$ family members in enhancing this disease.

Interleukin-15 (IL-15)

IL-15 is a 13kDa protein that is structurally similar to IL-2 and is produced by a variety of cells types, ranging from innate cells such as monocytes or dendritic cells to epithelial cells of the kidney, lung, heart, muscle and skin (39). IL-15 typically signals through a heterotrimeric receptor that consists of a unique IL-15Rα (CD215) subunit, the shared IL-2/15Rβ subunit, and the γ$_c$ subunit. The IL-15Rα is expressed by lymphoid cells (including CD8$^+$ T lymphocytes, NK Cells and γδ T lymphocytes), myeloid cells (including monocytes, macrophages, and dendritic cells), and nonhematopoietic cells (including colonic epithelial cells, microglial cells, or keratinocytes) (40). As discussed earlier (in the section on IL-2), expression of the β/γ$_c$ subunits are often restricted to T lymphocytes or NK cells. IL-15 binds with highest affinity to the IL-15Rα ($K_d \sim 10^{-11}$ M), which can then associate with the remaining β/γ$_c$ subunits that are expressed on the same cell, known as cis-presentation (40). Interestingly, trans-presentation can also occur, in which a cell expressing the IL-15Rα subunit binds IL-15 and then presents it to another nearby cell expressing only the β and γ$_c$ subunits to initiate IL-15-mediated signaling of the recipient cell. Of note, trans-presentation does not occur with IL-2, likely due to the lower binding of affinity of IL-2 with IL-2Rα (27). The IL-15/IL-15Rα complex has been shown to undergo endosomal recycling in certain cells, such as monocytes, which is a distinguishing feature

from IL-2 (41). The high-affinity complex for IL-15 signaling involves engagement of all three (α/β/γ_c) subunits. However, IL-15 can also signal through an intermediate-affinity complex consisting of only the latter IL-2/15Rβ and γ_c subunits ($K_d \sim 10^{-9}$ M); as mentioned earlier, this results in competition of this receptor complex with IL-2 due to the redundant subunits (40). While the IL-15Rα subunit is not known to engage any JAKs, the remaining two subunits make use of JAK/STAT signaling networks to influence cellular actions. The shared IL-2/15Rβ subunit binds JAK1 leading to STAT3 or STAT5 activation, while the γ_c subunit binds JAK3 and leads to STAT5 activation. The STAT3/5 heterodimers or STAT5 homodimers lead to transcription of the anti-apoptotic gene *BCL2* and the proto-oncogenes *MYC*, *FOS*, and *JUN*, among others (42). IL-15 signaling is known to support CD8^{+} memory T cell survival, drive the expansion and maintenance of T cells and NK cells, and also enhance CD8^{+} T cell production of granzymes, IFNγ, and tumor necrosis factor α (42).

AA patients demonstrated increased levels of serum IL-15 compared to healthy controls, and patients with more severe forms of AA (AT or AU) exhibited significantly more IL-15 in comparison to patients with a few patches of hair loss (43, 44). Additionally, SALT scores, an AA clinical scoring system based on percentage of hair loss on the scalp, positively correlated with levels of circulating IL-15 (45). IL-15 levels in the serum have also been observed to correlate with disease duration (46). In comparison to healthy controls, both AA patients and AA mice have increased expression of IL-15 and IL-15Rα in the outer root sheath of the hair follicle (7). It is hypothesized that expression of the IL-15/IL-15Rα complex by skin cells engages the β/γ subunits on infiltrating NKG2D^{+} CD8^{+} T cells and enhances their IFNγ production. This local increase in IFNγ can then act back on the skin epithelium to enhance expression of IL-15 and expression of NKG2D ligands, leading to further recruitment/activation of effector CD8^{+} T cells. This IL-15/IFNγ feedback loop between CD8^{+} T cells and the epithelium surrounding the hair follicle is thought to be a driving force to the diseased state (7). This concept was further supported using the murine skin graft induction model, in which mice treated with IL-2/15Rβ blocking antibody were protected from disease, had significantly reduced numbers of skin-infiltrating NKG2D^{+} CD8^{+} T cells, failed to upregulate MHC molecules in the skin and presented a skin gene signature similar to an unaffected mouse (7). Overall, these findings suggest that IL-15 may contribute to ongoing disease in patients, likely by enhancing effector functions of pathogenic CD8^{+} T cells.

JAK inhibition

JAKs phosphorylate their target substrates by transferring the terminal phosphate group from a high-energy adenosine triphosphate (ATP) molecule to the hydroxyl (-OH) residue of a tyrosine. This occurs when an ATP molecule binds to an open site within the ~300 base pair kinase domain of the active JAK. This ATP binding pocket is surrounding by the N-terminal lobe and the C-terminal lobe of the kinase domain. The N lobe helps to orient and anchor the ATP, while the C lobe binds the ATP and initiates the phospho-transfer (47). Interest in JAK inhibition as a therapeutic strategy was invigorated in 2005 when patients with myeloproliferative disorders (MPD) were found to have a JAK2 point mutation leading to its constitutively active state (48). This spurred the development of an inhibitor with activity against JAK2, and in 2011 the FDA approved the use of ruxolitinib for the treatment of patients with MPD. Shortly after, in 2012, tofacitinib was approved for patients with rheumatoid arthritis that were resistant to other treatments (49). JAKis are small membrane-permeable molecules that work by outcompeting ATP for binding in the pocket of the kinase domain, which ultimately prevents the JAK from phosphorylating its target substrate. However, due to similarities in the structure of the ATP-binding sites across the JAKs, the first generation of JAKis inhibit more than one JAK, typically in a hierarchical order based on binding site affinity. Second generation JAKis have been developed to more selectively inhibit individual JAK family members, with many currently being tested in clinical trials for a variety of autoimmune diseases (50). The preclinical rationale and the first demonstration of efficacy for JAKis to treat AA were published in 2014 (7, 51); these publications heralded the explosion in interest and clinical trials examining the efficacy and safety of JAKis for patients with AA as well as other dermatological conditions. Select clinical trials are described in Table 2.

First generation JAK inhibitors

The first generation of JAKis affect more than one JAK family member and have shown promising results in the context of AA; the efficacy of first generation JAKis in AA may be due to the inhibition of multiple cytokines/JAK signaling pathways that are contributing in parallel, and are partially redundant, in disease pathogenesis. The first generation of JAKis are comprised of ruxolitinib, tofacitinib, baricitinib, and oclacitinib (52). Of this group, the first three have been explored as treatments for AA and will be discussed further.

Ruxolitinib

Ruxolitinib is commonly regarded as a JAK1/JAK2 inhibitor (Table 1). The activity profile of ruxolitinib makes it an interesting therapeutic candidate for AA given how it can dampen both γ_c family cytokine signaling (JAK1/JAK3) and also IFNγ signaling (JAK1/JAK2). Thus, ruxolitinib is likely to act upon immune cells,

TABLE 1   JAK inhibitors with documented efficacy for treating AA.

| | IC$_{50}$ (nM) | | | |
	JAK1	JAK2	JAK3	TYK2
Tofacitinib (53)	15	71	45	472
Ruxolitinib (54)	3.3	2.8	428	19
Baricitinib (53)	0.78	2	253	14
Abrocitinib (55)	29	803	>10000	1253
Delgocitinib (56)	2.6	2.8	13	58
Upadacitinib (53)	0.76	19	224	118
Brepocitinib (57)	23	17	77	6494
Ritlecitinib (58)	>10000	>10000	33.1	>10000

IC$_{50}$ values denote the concentration of drug that reduces enzymatic activity of each JAK member by 50%. All reported assays performed in the presence of 1 mM ATP.

which are responsive to γ_c family cytokine signaling, and act at the level of the hair follicle, which is responsive to IFNγ signaling. Ruxolitinib was among the first of the JAKis that demonstrated efficacy for AA. In 2014, three patients with moderate/severe AA treated with oral ruxolitinib exhibited nearly complete hair regrowth after 3-5 months of treatment. Ruxolitinib treatment in these patients resulted in fewer CD8$^+$ and CD4$^+$ T cells infiltrating the hair follicle and its periphery (7). The positive outcome from this initial study led to numerous investigations testing the efficacy of ruxolitinib further.

From 2016 onward, case reports have appeared highlighting the use of ruxolitinib to treat AA, sometimes under special circumstances such as AA patients presenting with additional autoimmune diseases as well as in pediatric AA patients. An early case report demonstrating ruxolitinib efficacy for AA involved a patient presenting with both AA and vitiligo, a cutaneous autoimmune disease that causes depigmentation of the skin and, like AA, is mediated by CD8$^+$ T cells and IFNγ. The patient received treatment for 20 weeks, and experienced significant regrowth of hair (85% of scalp coverage from a baseline 63% coverage) after only 12 weeks of treatment, and this regrowth was maintained for at least 12 weeks after treatments ceased (59). A more recent case report demonstrated one of the first uses for ruxolitinib to treat AA in a preadolescent patient. A 9-year-old patient with AT was treated twice daily with 20 mg of oral ruxolitinib, and 4 months of treatment yielded nearly complete regrowth of hair on scalp and eyebrows. The dosage was tapered to 10 mg every other day, a level that maintained efficacy with no adverse effects (60).

Early cases reports identified that hair regrowth after cessation of ruxolitinib is not sustained long-term, as patients tended to experience hair loss relapse after ending treatment. Case reports of ruxolitinib for treating AA have involved testing different treatments regimens, such as changing the duration and altering dosage, to prolong positive outcomes. A report involving two patients, one presenting with AT and one

presenting with AU, were given daily ruxolitinib treatments for 13-14 months, and then tapered to less frequent dosing. The patients exhibited near complete regrowth by 6-8 months of treatment, and the tapered dosing led to maintenance of hair regrowth (61). An additional study demonstrated efficacy of lower dosing ruxolitinib to reverse hair loss in severe AA patients. Eight patients were treated twice daily with 10-25 mg of ruxolitinib for a range of 5-31 months. Interestingly, six of the enrolled patients had undergone prior tofacitinib therapy with varied results. Five of the enrolled patients attained near complete hair regrowth, with four of them having received the lower dosage of 10 mg ruxolitinib twice daily (62).

A few clinical trials testing the use of both systemic oral ruxolitinib and localized topical ruxolitinib to treat AA have been completed. A phase 2, open-label trial with oral ruxolitinib involved a cohort of twelve patients with moderate/severe AA that received daily ruxolitinib for a duration of 3-6 months. 9 of 12 patients had at least 50% regrowth of hair and continuous reduction in SALT scores over time while receiving treatment. Three months after treatment cessation, all nine responsive patients noted hair shedding, with three of those patients experiencing marked hair loss. Gene expression profiling revealed that ruxolitinib treatment normalized the aberrant IFNγ and CD8$^+$ T cell-based gene signatures in the scalp, demonstrating that AA lesion biopsies taken after 12 weeks of treatment clustered closer to healthy skin biopsies than baseline AA biopsies (Table 2) (64).

A phase 2, double-blind, placebo-controlled trial examining topical ruxolitinib cream was conducted in a cohort of 16 AU patients that received twice daily ruxolitinib applications for 12 weeks. 5 of 16 patients exhibited partial regrowth in areas treated with topical ruxolitinib. However, hair loss was noted after cessation of treatments at 6-week and 12-week follow-up time points (75). Soon after, another phase 2, double-blind, vehicle-controlled trial appraised topical ruxolitinib cream in a cohort of 78 patients with moderate AA. Patients received daily

TABLE 2 Completed or currently active clinical trials involving the use of JAK inhibitors to treat AA.

JAK inhibitor	Study type	Patient information	Dosing	Outcome	End Date	Trial ID	References
Tofacitinib	pilot study, open label	66 adult patients with severe AA, ophiasis, AT, or AU	5 mg twice daily for 3 months	• 42 of 66 patients were responsive. 21 of the responsive patients reached a $SALT_{50}$ score • Greatest reduction in SALT scores observed in patients with AA (70%) and ophiasis (68%) • 20 patients were followed for 3 months after Tx cessation, and all experienced hair loss • No serious adverse effects (AE) reported	August 2015	NCT02312882 NCT02197455	Kennedy Crispin et al. (63)
Ruxolitinib	Phase 2, open label	12 adult patients with moderate to severe AA	20 mg twice daily for 12-24 weeks	• 9 of 12 patients were responsive, with an average of 92% regrowth • Mean SALT scores of responders: Baseline (65.8), 3 months Tx (24.8), 6 months Tx (7.3) • 3 months after cessation all 9 responders noted hair shedding, with 3 experiencing marked hair loss • No serious AE	April 2016	NCT01950780	Mackay-Wiggan et al. (64)
Delgocitinib ointment (LEO 124249)	Phase 2, double blind, vehicle controlled	31 adult patients with moderate to severe AA (>30% scalp involvement), randomly assigned	30 mg/g ointment applied twice daily (20) or vehicle control (11) for 12 weeks	• The primary outcome measured was change in SALT score. The mean change after 12 weeks of treatment was a decrease of 3.8 in the drug group, and a decrease of 3.4 in the vehicle group. • No serious AE	December 2016	NCT02561585	Mikhaylov et al. (65)
Ruxolitinib cream	Phase 2 Part A: open label Part B: double-blind, placebo controlled, followed with optional open label extension	Part A: 12 adult patients with moderate/severe AA Part B: 78 adults with moderate to severe AA randomly assigned to ruxolitinib (39) or vehicle (39) group Extension: 63 patients, 31 from ruxolitinib group and 32 from vehicle	Part A: 1.5% topical ruxolitinib cream, twice daily for 24 weeks Part B:1.5% topical ruxolitinib cream or vehicle twice daily for 24 weeks Extension: 1.5% topical ruxolitinib, twice daily for 24 weeks	Part A: 6 of 12 patients reached a $SALT_{50}$ score (50% or greater improvement in SALT score) after 24 weeks of Tx. Part B: 5 of 39 patients in both the Ruxolitinib cream group and the placebo group reached a $SALT_{50}$ score. Extension: 3 patients from previous ruxolitinib group reached a $SALT_{50}$ score, and 1 patient reached a $SALT_{90}$ score. 4 patients from previous vehicle group reached a $SALT_{50}$ score. • Serious AE experienced by 1 patient in Part A, and 3 patients in Part B	October 2017	NCT02553330	Olsen et al. (66)
Tofacitinib (Xeljanz)	Phase 2, open label	12 adult patients with moderate to severe AA, AT, or AU	5 mg-10 mg twice daily for 6 months with option to extend up to 18 months.	• 11 of 12 patients showed SALT score improvement. Mean SALT score of 81.3 at baseline dropped to 40.8 at the end of treatment. • 8 of 12 patients reached a $SALT_{50}$ score and were followed for 6 months after Tx cessation. 6 of the 8 experienced hair loss after stopping and 1 patient maintained hair regrowth during this period. • 1 patient experienced AE resulting in discontinuation of Tx	December 2017	NCT02299297	Jabbari et al. (67)
Tofacitinib ointment	Phase 2, open label	10 adult patients with AA (at least 2 patches), AT or AU	2% topical tofacitinib, twice daily to half of affected area for 6 months	• 3 of 10 patients were responsive, with 1 patient experiencing significant regrowth and 2 exhibiting partial regrowth. • Mean SALT score decrease of responsive patients was 34.6% • No serious AE	July 2018	NCT02812342	Liu et al. (68)
Ritlecitinib (PF-06651600)	Phase 2a, double blind with optional	72 adult patients with moderate to severe AA. Randomly	200 mg daily for 4 weeks, then 50 mg daily for 20 weeks or placebo	• At 24 weeks of Tx, 50% of patients achieved $SALT_{30}$ scores, and 25% had achieved $SALT_{90}$ scores by this time. • No serious AE	May 2019	NCT02974868	King et al. (69)

(Continued)

TABLE 2 Continued

JAK inhibitor	Study type	Patient information	Dosing	Outcome	End Date	Trial ID	References
	single blind extension	assigned to ritlecitinib (48) and placebo (24)					
Brepocitinib (PF-06700841)	Phase 2a, double blind with optional single blind extension	70 adult patients with moderate to severe AA. Randomly assigned to brepocitinib (47) and placebo (23)	60 mg daily for 4 weeks, then 30 mg daily for 20 weeks or placebo	• At 24 weeks of Tx, 64% of patients achieved $SALT_{30}$ scores, and 34% had achieved $SALT_{90}$ scores by this time. • 2 patients receiving brepocitinib experienced serious AE	May 2019	NCT02974868	King et al. (69)
ATI-501 *JAK1/JAK3 inhibitor*	Phase 2, double blind, placebo controlled	87 adult patients with AA, AU, or AT, randomly assigned	400 mg (23), 600 mg (23), 800 mg (22) or placebo (19) taken daily for 24 weeks	• The primary outcome was the percent change in SALT scores. After 24 weeks, changes were: -25.6 (400 mg), -30.4 (600 mg), -25.9 (800 mg), and -6.3 (placebo). • No serious AE	June 2019	NCT03594227	(70)
Ritlecitinib (PF-06651600)	Phase 2B/3, double blind, placebo controlled and dose ranging	718 adult and adolescent (> 12 years old) patients with moderate to severe AA (≥ 50% scalp hair loss)	Range of daily oral dosing, broken into a 4-week loading phase/20-week maintenance phase/24-week extension phase: •200mg/50mg/50mg •200mg/30mg/30mg •50mg/50mg/50mg •30mg/30mg/30mg •10mg/10mg/10mg •placebo	• Primary endpoint was a SALT score ≤ 20. The three highest treatment groups (31%, 22%, and 24% respectively) resulted in the most patients reaching that endpoint. • 30 mg group resulted in 14.5% of patients reaching endpoint, while the 10 mg group only had 2% of patients reach endpoint. • 12 patients, dispersed throughout test groups, experienced serious AE	December 2020	NCT03732807 (ALLEGRO-2b/3)	(71)
Baricitinib (LY3009104)	Phase 3, double blind placebo controlled	654 adult patients with severe AA. (>50% scalp involvement)	4 mg twice daily baracitinib (281), 2 mg twice daily baricitinib (184), or placebo (189)	• The primary outcome was to achieve a SALT score of 20. 38.8% of patients in the 4 mg achieved the outcome, along with 22.8% in the 2 mg group and 6.2% in the placebo group. • Serious AE reported in 13 patients, dispersed throughout all groups	January 2021	NCT03899259 BRAVE-AA2	King et al. (72)
Baricitinib (LY3009104)	Phase 2/3, double blind, placebo controlled	546 adult patients with severe AA (>50% scalp involvement), randomly assigned	4 mg twice daily baricitinib (234), 2 mg twice daily baricitinib (156), or placebo (156)	• The primary outcome was to achieve a SALT score of 20. In the 4 mg group, 35.9% of patients achieved the outcome, along with 19.4% in the 2 mg group and 3.3% in the placebo group. • Serious AE reported in 15 patients, dispersed throughout all groups	February 2021	NCT03570749 BRAVE-AA1	King et al. (72)
CTP-543	Phase 3, double blind, placebo controlled	706 adult patients with severe AA (≥ 50% scalp hair loss), randomly assigned	12 mg CTP-543, 8 mg CTP-543, or a placebo taken twice daily for 24 weeks	• The primary outcome was to achieve a SALT score ≤ 20. 42% of patients in the 12 mg group achieved the outcome, along with 30% in the 8 mg group and 1% in the placebo group. Significant differences noted as early as 8 weeks into Tx. • Serious AE reported in 9 patients dispersed throughout all groups	May 2022	NCT04518995 THRIVE-AA1	(73)
CTP-543	Phase 3, double blind, placebo controlled	517 adult patients with severe AA (≥ 50% scalp hair loss), randomly assigned	12 mg CTP-543, 8 mg CTP-543, or a placebo taken twice daily for 24 weeks	• The primary outcome was to achieve a SALT score ≤ 20. 38.3% of patients in the 12 mg group achieved the primary outcome, along with 33% of patients in the 8 mg group and 0.8% in the placebo group. • Serious AE were experienced by 5 patients in the trial.	June 2022	NCT04797650 THRIVE-AA2	(74)
CTP-543	Phase 2, double blind,	300 adult patients with severe AA (≥ 50%	Part A: 12 mg or 8 mg twice daily for 24 weeks, followed	Primary outcome measures: Part A: frequency of patients exhibiting loss of maintenance upon dose reduction	Est. Oct 2022	NCT04784533	-

(Continued)

TABLE 2 Continued

JAK inhibitor	Study type	Patient information	Dosing	Outcome	End Date	Trial ID	References
	placebo controlled	scalp hair loss), randomly assigned	by 24 weeks of dose reduction or placebo. Part B: 12 mg or 8 mg twice daily for 24 weeks for patients experiencing loss of maintenance during Part A.	or drug discontinuation. Part B: frequency of patients experiencing restoration of regrowth			
Ritlecitinib (PF-06651600)	Phase 3, open label	1049 adult and adolescent (> 12 years old) patients with AA. Some patients have participated in prior PF-066511600 clinical trials.	Naïve patients: 200 mg daily for 1 month, then 50 mg daily for 35 months Previously enrolled patients: 50 mg daily for 36 months	Number of subjects reporting adverse events, abnormal vital signs, or abnormal clinical lab values.	Est. July 2024	NCT04006457 - **ALLEGRO-LT**	

applications to affected areas for a duration of 24 weeks. Both the ruxolitinib cream and vehicle treated groups resulted in 5 of 39 patients showing reduced SALT scores over the 24-week period. This study concluded that topical ruxolitinib does not significantly affect AA patients (Table 2) (66).

To accompany the numerous case reports and trials, the protective effects of ruxolitinib have been demonstrated using an *in vitro* hair follicle culture model. Human dermal papilla cells (hDPCs) pre-treated with ruxolitinib had significantly lower MHC Class II expression following IFNγ exposure when compared to resting hDPCs exposed to IFNγ. Furthermore, hDPCs treated with ruxolitinib showed a reversal in the expression of IFNγ-inducible genes (such as caspase-1, IL-15, IL-1β) following stimulation with IFNγ. These findings suggest that ruxolitinib plays a role in helping maintain an immune privileged state of the hair follicle, likely by dampening the downstream effects of IFNγ signaling, in addition to its effects on immune effectors (76).

Of note, a deuterated form of ruxolitinib, CTP-543, has recently been developed for the treatment of AA. Efficacy for CTP-543 has been supported by data from a phase 2, double-blind, placebo-controlled trial involving 149 adult patients with severe AA that received 4 mg, 8 mg, or 12 mg of CTP-543 twice daily or a placebo for a duration of 24 weeks. The primary endpoint was the frequency of patients achieving a $SALT_{50}$ score, which is a 50% or greater reduction in SALT scores in comparison to baseline. The trial found that patients in the higher dosage groups (8 mg and 12 mg) had significantly more patients reach the endpoint (47% and 58%) in comparison to placebo (9%). The frequency of patients experiencing adverse effects were similar among all treatment groups, and only one

patient (12 mg dosage) experienced a serious adverse effect (77). The positive outcomes from this trial led to the initiation of two larger-scale studies, THRIVE-AA1 and THRIVE-AA2, examining further efficacy and safety (Table 2). THRIVE-AA1 contained 706 patients receiving 12 mg CTP-543, 8 mg CTP-543 or placebo twice daily for 24 weeks, with a primary endpoint of achieving a SALT score ≤ 20. 42% of patients in the 12 mg group and 30% in the 8 mg group met this endpoint, compared to 1% of the placebo group. Additionally, 53% of patients in the 12 mg group and 42% in the 8 mg group were satisfied or very satisfied with their results after 24 weeks of treatment compared to 5% in the placebo group. Serious adverse effects were reported by 1 patient receiving 12 mg, 4 patients receiving 8 mg, and 4 patients in the placebo group (73). THRIVE-AA2 contained 517 patients randomly assigned to 12 mg CTP-543, 8 mg CTP-543, or placebo. Similar to THRIVE-AA1, the primary endpoint was to achieve a SALT score of ≤ 20. 38.3% of patients in the 12 mg group and 33% of patients in the 8 mg group met this endpoint, compared to 0.8% in the placebo group. 52% and 47% of patients in the 12 mg and 8 mg groups were satisfied or very satisfied with their results compared to 2% of patients in the placebo group (74). Together, the two THRIVE trials enrolled over 1200 patients. The positive results from these massive trials indicate that CTP-543 may be the next inhibitor granted approval for treating adult patients with AA.

Tofacitinib

Tofacitinib is typically classified as a pan-JAK inhibitor with preference towards the JAK1/JAK3 pair (Table 1). The

inhibitory properties of tofacitinib show it to be effective in dampening signals from the γc family cytokines (JAK1/JAK3) and IFNγ (JAK1/JAK2), indicating that it can act on immune cells and on the hair follicle. To date, tofacitinib has likely been prescribed for patients with AA more than any other JAKi.

Tofacitinib efficacy for AA was initially suggested in 2014, in which a patient presenting with both plaque psoriasis and AU exhibited complete regrowth of scalp hair after 3 months of treatment and full regrowth of all body hair after 8 months of treatment (51). Since then, many case studies have appeared supporting the efficacy of tofacitinib to treat AA. An early report involved a patient with persistent AA who received daily tofacitinib for 4 months and demonstrated near complete regrowth of hair. Transcriptional analysis of skin biopsies taken 4 weeks into treatment revealed a decrease in IFN and cytotoxic T cell gene signatures when compared to baseline biopsies. Additionally, serum levels of Cxcl10, an IFN-inducible chemokine, were noticeably lower after 4 weeks of tofacitinib treatment. However, upon ceasing treatment, the patient presented with near complete loss of hair at the 16-week timepoint (78). A more recent entry in the literature reported the efficacy of treating a patient that presented with AU, atopic dermatitis, and ulcerative colitis simultaneously. Daily tofacitinib treatments led to marked scalp hair regrowth by 4 months of treatment, along with significant improvement in itch and erythema, and colonic biopsies suggested a remissive state. The treatment dose for this patient was tapered, and, at the 10-month follow-up, all three disease states remained well controlled. This report highlights a potential advantage of pan-JAKi therapy for patients exhibiting multiple diseases of inflammatory nature (79).

There have also been ample reports promoting the efficacy of tofacitinib to treat AA in the pediatric population, a group with increased risk of developing more severe and chronic forms when compared with adults (80). Increasing the pool of safe and effective therapeutics for pediatric patients would be especially beneficial given the psychological burdens this disease carries in children (81). An initial report involved 4 patients (5-7 years of age) with AT or AU who were treated with oral tofacitinib. Drug dosing was based on concurrent clinical trials testing tofacitinib in juvenile idiopathic arthritis patients. Two AA patients demonstrated complete regrowth of hair by 3-6 months of daily treatments, another had 62% of regrowth occur, and the remaining patient exhibited no substantial regrowth (82). Shortly after, another case series was communicated detailing 11 patients (7-11 years of age) that exhibited a range of AA, from involving eyebrows only to AU. The patients received daily oral tofacitinib for at least 6 months, starting at a relatively low dose (2.5-7.5 mg daily), which was increased depending on patient tolerance and responsiveness. Nine of the patients responded well, with a mean SALT score change of 67.8%, and two remaining patients experienced no noticeable hair regrowth. The treatment appeared well tolerated, with most adverse events being characterized as mild and transient (83). These studies support that systemic tofacitinib therapy may be an effective option for treating pediatric AA.

A few clinical trials investigating the efficacy of daily oral tofacitinib to treat AA have been completed. An open-label trial was conducted with 66 patients, with the majority (71.2%) having AU at the time of enrollment. After 3 months of treatment, 32% of the patients had strongly responded by exhibiting significant improvements in SALT scores (>50% change), and the treatment was tolerated with limited adverse effects. Immunofluorescent staining of a biopsy from a strongly responding patient had a marked reduction in STAT3 expression within the follicular epithelium after 2 months of treatment, suggesting that blocking this pathway is associated with a positive outcome. 20 patients were evaluated at 3 months after cessation of treatment, and these patients had all experienced hair loss, suggesting that transient treatment with tofacitinib does not result in a durable response (Table 2) (63). Another open-label trial examining tofacitinib for AA involved 12 patients. Eleven patients completed the study, having received daily treatments over 6-18 months. 8 of 11 patients reached the primary efficacy endpoint of a SALT score reduction of > 50%. Upon cessation, six of the eight responding patients experienced hair loss as early as 4 weeks post treatment, while one patient presented with maintained hair regrowth even up to 6 months post treatment (Table 2) (67).

The use of tofacitinib as a topical therapeutic agent has also been investigated. An initial study examined the efficacy of topical 2% tofacitinib to treat three pediatric patients exhibiting patchy AA or AT. They received twice daily applications, and 2 of 3 patients (one with patchy AA and the other with AT) experienced 80% and 95% regrowth of scalp hair. However, a third patient with AT failed to exhibit any regrowth (84). Shortly after, a clinical trial was completed which tested the efficacy of daily application of 2% tofacitinib ointment in ten adult patients with moderate/severe AA (≥ 2 patches of scalp hair loss). 3 of 10 patients had hair regrowth occur, with an average SALT score change of 34.6% and minimal adverse effects (Table 2) (68). Larger, placebo-controlled studies are needed to confirm the benefits for topical delivery of tofacitinib, and, although the data above appear to show an efficacy signal, improved topical delivery methods will likely be required. Overall, however, there is substantial support for the use of systemic tofacitinib as a therapeutic option for patients with AA.

Baricitinib

Baricitinib is considered a dual JAK1/JAK2 inhibitor given the similarly strong binding interactions with those two proteins (Table 1). Efficacy of baricitinib for AA was initially suggested in 2015, when a CANDLE patient was being treated with this drug; the patient exhibited comorbid AA and demonstrated complete

hair regrowth after 9 months of treatment (85). This clinical data was further supported by studies done in the murine model of AA in the same report. Systemic administration of baricitinib led to reduced frequency of mice developing disease, reduction of CD8^{+} T cell infiltrates and a reduction of MHC Class I and Class II expression in the skin, suggesting baricitinib may help prevent the collapse of an immune privileged state of the hair follicle. Additionally, topical baricitinib applied onto lesions of AA mice led to nearly complete regrowth of hair (85). A later published case-report further indicated efficacy for baricitinib in the treatment of AA. This report involved an adult patient with progressive AT who received daily oral baricitinib. She was initially unresponsive to a starting dose of 2 mg; however, upon increasing the dosage to 4 mg, she experienced remarkable hair regrowth. 8 months of treatment resulted in 97% of regrowth on the scalp as well as regrowth on eyebrows and eyelashes. After 13 months of treatment, regrowth had been maintained, and no adverse effects were noted (86).

In the past few years, larger clinical trials testing baricitinib to treat AA have been completed. A phase 2, double blind, placebo-controlled trial randomized 110 patients with severe AA into four test groups: placebo, 1 mg, 2 mg, or 4 mg of baricitinib daily. After 12 weeks of treatments, interim SALT scores were assessed, and the 2 mg and 4 mg groups presented with the highest frequency of patients achieving $SALT_{30}$ scores (29.6% and 33.3% respectively) (87). These two higher treatment groups were selected for continued usage, with patients initially assigned to the 1 mg dosing being transitioned to a 4 mg dosage for the remainder of the trial. A later interim analysis at 36 weeks of treatment aimed to identify the frequency of patients with SALT scores ≤ 20. The 2 mg and 4 mg groups had significantly higher frequencies of patients, 33% and 51.9% respectively, meet the criteria in comparison to the placebo group(3.6%) (Table 2). Overall, this study demonstrated that longer durations of treatment with higher dosages of baricitinib were effective at stimulating scalp hair regrowth and was well tolerated, with most adverse effects being classified as mild (87).

The positive results from the previous trial spurred the formation of two large-scale phase 3 trials looking at 2 mg or 4 mg of oral baricitinib to treat severe AA. The two trials, termed BRAVE-AA1, and BRAVE-AA2 contained 654 and 546 patients, respectively. Each trial randomly assigned patients to daily treatments consisting of placebo, 2 mg, or 4 mg of baricitinib, with a primary outcome of achieving a SALT score of ≤ 20. After 36 weeks of treatment, the 4 mg group had 35.9-38.8% of patients achieve the primary outcome, while the 2mg group had 19.4-22.8% and the placebo had only 3.3-6.2% of patients reach the primary outcome (Table 2) (72). Favorable results from the BRAVE-AA1 and BRAVE-AA2 trials led to the first-in-disease systemic FDA-approved treatment for AA. On June 13th 2022, the FDA approved a once-daily regimen of baricitinib (Olumiant) available as 1 mg, 2 mg, or 4 mg tablets for adults with severe AA (88). Approved FDA labeling of Olumiant

includes boxed warnings for potential serious adverse effects. This moment marks an important time in history for AA patients and their clinicians alike who now have a more accessible treatment option available. This is especially crucial given how insurance approval has historically been an issue for AA patients seeking off-label usage of JAKis (89).

Second generation JAK inhibitors

Second generation JAKis were designed to specifically inhibit a single JAK family isoform, which may allow for more selective intervention of inflammatory diseases based on the contributing cytokines and their respective JAK signaling molecules. In addition, their more limited range of inhibitory effects may reduce the potential for adverse events. Despite their relatively recent development, studies have already begun to emerge describing efficacy for the resolution of hair loss in AA patients. The results from select studies will be discussed further below.

Abrocitinib

Abrocitinib (PF-04965842) is a JAK1-specific inhibitor that has shown efficacy in treating patients with atopic dermatitis (AD), which is a disease marked by a higher risk of developing AA (Table 1) (90). A 2022 case report demonstrated efficacy of abrocitinib for AA, when a patient presenting with both AD and AU began a daily oral regimen of 200 mg. Within 12 weeks, patchy hair regrowth was noted, and full regrowth of scalp hair was observed after one year of treatment. The patient's AD lesions were noticeably improved as well, suggesting that this selective JAKi may be a promising option for patients with dual AD and AA diagnoses (91). Another recent report highlighted two AD patients presenting with severe AA that had noticeable hair-regrowth occur upon extended duration of abrocitinib therapy. Both patients were enrolled into a long-term clinical trial termed JADE EXTEND after showing responsiveness to abrocitinib, noted by improvement in AD, in an initial shorter treatment window. The patients had noticeable hair regrowth occur at 12-14 weeks into the trial that maintained throughout the course of treatment (92).

Upadacitinib

Upadacitinib (ABT-494) is a JAK1-specific inhibitor which was initially shown to be effective in treating AD (Table 1) (93). A case report describing two patients with AD and comorbid AA revealed potential efficacy of upadacitinib for treating AA. One patient had AU (SALT score 100), while the other patient had a resistant patch of AA (SALT score 43). Near complete hair

regrowth was observed for both patients after 4 months of treatment with 30 mg daily (94). A more recent case report again indicated efficacy in treating a patient presenting with dual AD and AA (SALT score 89.2). The patient had failed multiple therapies for both diseases, but upon switching to 30mg of daily upadacitinib, the patient demonstrated remarkable clinical improvements in both AD and AA, with no reported adverse effects. Larger, placebo-controlled studies will be needed to determine true efficacy in patients with AA (95).

Brepocitinib

Brepocitinib (PF-06700841) is an inhibitor of JAK1 and TYK2 that was initially shown to be effective in treating patients with plaque psoriasis (Table 1) (96). Efficacy for brepocitinib for treating AA was demonstrated in the recently reported ALLEGRO trial, where it was tested in parallel with ritlecitinib, which is discussed further below. 47 patients with severe AA were treated daily with 60 mg of oral brepocitinib for 4 weeks, and then tapered to a 30 mg daily dose for 20 additional weeks. After 24 weeks of treatment, 64% of patients achieved the primary outcome of a $SALT_{30}$ score, which is a 30% or greater reduction in SALT scoring in comparison to baseline. Two patients discontinued use after experiencing serious adverse effects (69). Lesional biopsies taken from patients receiving brepocitinib (or ritlecitinib) at the 12- and 24-week timepoints revealed significant decreases in $CD3^+$ and $CD8^+$ T cell counts in comparison to baseline levels. Transcriptomic analysis from biopsies also showed a significant decrease in inflammatory gene signatures (immune genes, Th1, Th2 and IL-12/IL-23) in the patients receiving either brepocitinib or ritlecitinib when compared to placebo (Table 2) (97). The positive outcome from this initial trial warrant further investigations into the efficacy of brepocitinib for treating AA, although it appears that further investigation and development by the manufacturer have focused on ritlecitinib over brepocitinib for the treatment of AA.

Ritlecitinib

Ritlecitinib (PF-06651600) is an inhibitor specific for JAK3 and the tyrosine kinase expressed in hepatocellular carcinoma (TEC) family of kinases (Table 1) (98). It acts through irreversible, covalent interactions with the cysteine 909 residue found on the active site of these kinases. The remaining members of the JAK family all possess a serine residue at this site, which accounts for the selectivity of ritlecitinib for JAK3 (58). This feature is likely clinically relevant given the use of JAK1, JAK2, and TYK2 broadly by wide-ranging receptors responsible for blood and tissue homeostasis and protective responses against pathogens. Additionally, targeting JAK3

spares signaling of immunoregulatory cytokines such as IL-10R (TYK2/JAK1), IL-27R (JAK1/JAK2), and IL-35R (JAK1/JAK2) (99), whose activity may contribute to the prevention of autoimmunity to the hair follicle, potentially making a selective JAK3 inhibitor a more potent strategy than pan-JAK inhibition. As previously mentioned, JAK3 associates exclusively with receptors for γ_c cytokines; because downstream signaling from γ_c receptors are most often transmitted in cells of the immune system, ritlecitinib represents a JAK inhibitor that may act specifically through the modulation of the immune compartment without directly influencing the hair follicle itself.

Ritlecitinib is the only irreversible JAKi being assessed for clinical use for AA patients. Efficacy of ritlecitinib for AA was demonstrated during a recently reported clinical trial (phase 2, double-blind, placebo-controlled) termed ALLEGRO, where a primary outcome was designated as a 30% improvement in SALT score. 48 patients with severe AA ($\geq$ 50% hair loss on scalp) were treated with a loading regimen of 200 mg daily of ritlecitinib for 4 weeks, and then treated with 50 mg daily for 20 weeks. By the 24th week of treatment, 50% of patients receiving ritlecitinib had reached the primary outcome, while only 3% of patients receiving placebo reached that endpoint. The number of patients experiencing adverse effects were similar between the groups; overall ritlecitinib was generally well tolerated by patients and may be an effective agent for restoring hair growth in patients with severe AA (69). Phase 3 and long-term extension trials are underway for ritlecitinib in patients with AA (Table 2).

Side effects of JAK inhibitors

While JAKis have demonstrated great promise for reversing the immune-mediated hair loss in AA, they also present a risk for potentially severe side effects. This notion is not necessarily surprising given the redundancy of JAK proteins for cytokine, growth factor, and hormone receptor signaling. Commonly documented minor adverse events experienced by AA patients taking JAKis include acne, headache, nausea, urinary tract infections, respiratory tract infections, anemia, thrombocytopenia, neutropenia, and elevated creatinine levels (100). Patients using JAKis also commonly exhibit elevated LDL levels, which is a known risk factor for cardiovascular disease (100, 101). Serious adverse effects most often reported in AA trial patients receiving JAKis include varicella zoster emergence, pneumonia, tuberculosis, sepsis, and the development of non-melanoma skin cancer (100).

Beyond AA, more extensive safety analyses have been reported for patients using JAKis to treat other diseases such as rheumatoid arthritis, atopic dermatitis, psoriatic arthritis, ulcerative colitis, and myeloproliferative disorders. Results from these studies should be carefully considered until more large-scale safety trials are completed specific for the AA

population. A recent meta-analysis comprised of over 126,000 JAKi patient cases for the above-mentioned diseases found that the most common adverse effects experienced were infections (herpes, influenza, fungal, and mycobacterial), thrombosis and pulmonary embolism, and neoplasms (102).

The FDA has issued boxed warnings to all JAK inhibitors that are being used for inflammatory diseases. These cautions constitute the highest level of safety warning assigned by the FDA, and are intended to alert consumers of major potential risks associated with the drug (103). In 2020, a large-scale phase-4 safety study compared the side effects of RA patients receiving 5-10 mg twice daily oral tofacitinib to patients receiving TNFα inhibitor injections for a mean duration of about 40 months. This robust study contained more than 1450 patients in each treatment group, and primary endpoints were to assess rate of cancers (excluding nonmelanoma skin cancer) and major adverse cardiovascular events (MACE) including death from cardiovascular causes, nonfatal stroke, and nonfatal myocardial infarction. A goal of this study was to examine whether tofacitinib met noninferiority criteria for the two primary endpoints; meeting this criterion indicates that there is no difference between the treatments based on the boundaries of the confidence intervals associated with the hazard ratio (104).

The incidence of cancers reported during follow-up appointments (median of 4 years), was 4.2% for tofacitinib users and 2.9% for TNFα users, with a hazard ratio of 1.48 (tofacitinib:TNFα). The most common cancer experienced among tofacitinib users was lung cancer, while breast cancer was most common among patients receiving the TNFα inhibitor. The frequency of tofacitinib users experiencing MACE was 3.4% compared to 2.5% of patients receiving TNFα inhibitor, with a hazard ratio of 1.33 (tofacitinib:TNFα). The most common MACE experienced was nonfatal myocardial infarction among tofacitinib users and nonfatal stroke among patients receiving the TNFα inhibitor. When compared to TNFα inhibitors, tofacitinib failed to meet noninferiority criteria for both incidence of cancers and MACE (104). The study determined that there is indeed a difference in adverse events experienced between users of tofacitinib and TNFα inhibitors. Of note, the efficacy was similar among all patients regardless of treatment, suggesting that in the context of RA, tofacitinib performs similarly to a well-documented biologic, but also presents a significantly greater safety risk (104).

Overall, JAKis have potential to cause off-target harm in addition to their therapeutic benefit. Thus, usage of JAKis should only be considered once the risks are clearly communicated to and comprehended by patients. Nevertheless, as more large-scale clinical trials are completed, more FDA approvals of JAKis to treat AA are likely in the not-too-distant future.

Conclusion

The past decade has been an exciting time for the identification and testing of new therapeutics for patients with AA. The therapeutic pool is changing from use of broad-acting immunosuppressants to more inferred and targeted approaches. These advances are possible due to the identification of novel disease mechanisms in basic science laboratories and pre-clinical AA animal models. Recent therapeutic approaches are targeting the actions of pro-inflammatory cytokines, which have been identified as crucial players and determinants of the onset, progression, and severity of AA. JAKis have taken the spotlight for now. However, despite the recent FDA approval of the first JAKi to treat AA, side effects and incomplete efficacy for refractory patients begs the question, "What else is there?" As more mechanisms of this enigmatic disease are unraveled, the arsenal of therapeutic options for AA will surely continue to grow.

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Conflict of interest

AJ has served as a consultant for and has received institutional payments for study-related costs from Pfizer.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Targeting inflammation and immune activation to improve CTLA4-Ig-based modulation of transplant rejection

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For the last few decades, Calcineurin inhibitors (CNI)-based therapy has been the pillar of immunosuppression for prevention of organ transplant rejection. However, despite exerting effective control of acute rejection in the first year post-transplant, prolonged CNI use is associated with significant side effects and is not well suited for long term allograft survival. The implementation of Costimulation Blockade (CoB) therapies, based on the interruption of T cell costimulatory signals as strategy to control allo-responses, has proven potential for better management of transplant recipients compared to CNI-based therapies. The use of the biologic cytotoxic T-lymphocyte associated protein 4 (CTLA4)-Ig is the most successful approach to date in this arena. Following evaluation of the BENEFIT trials, Belatacept, a high-affinity version of CTLA4-Ig, has been FDA approved for use in kidney transplant recipients. Despite its benefits, the use of CTLA4-Ig as a monotherapy has proved to be insufficient to induce long-term allograft acceptance in several settings. Multiple studies have demonstrated that events that induce an acute inflammatory response with the consequent release of proinflammatory cytokines, and an abundance of allograft-reactive memory cells in the recipient, can prevent the induction of or break established immunomodulation induced with CoB regimens. This review highlights advances in our understanding of the factors and mechanisms that limit CoB regimens efficacy. We also discuss recent successes in experimentally designing complementary therapies that favor CTLA4-Ig effect, affording a better control of transplant rejection and supporting their clinical applicability.

KEYWORDS

transplant rejection, costimulation-blockade, CTLA4-Ig, inflammation, immunological tolerance

1. Introduction

Long-term management of maintenance immunosuppression in organ transplantation remains complex. Calcineurin inhibitors (CNI)-based therapy, despite greatly decreasing acute rejection rates and improving 1-year outcomes, has not had as great effect on long-term allograft survival, and prolonged treatment is also associated with high risk of acute and chronic nephrotoxicity, post-transplant diabetes, elevated blood pressure, hyperlipidemia and neurotoxicity (1). Decades of research have shown that interfering with T cell costimulatory signals as a strategy to control undesired immune allo-responses, implementing the so called costimulation blockade (CoB)-based therapies, has the potential to induce tolerance to allogenic tissues and alleviate many of the unwanted side effects associated with current immunosuppressive therapies (2, 3). The only approach that has reached the clinic to date has been the use of cytotoxic T-lymphocyte associated protein 4 (CTLA4)-Ig, fusion protein that prevents the interaction of CD80 and CD86 on antigen presenting cells with CD28 on T cells. TCR stimulation without concomitant signaling through CD28 results in an abortive T cell activation and actuation of a program of apoptosis, induction of T cell anergy, and conversion into regulatory T cells (Treg) (4, 5).

Belatacept, a high-affinity version of CTLA4-Ig (6), was approved by the US Food and Drug Administration (FDA) for use in kidney transplant recipients in 2011 after evaluating results from the phase 3 randomized BENEFIT trials (7, 8). Despite its calcineurin-sparing benefits, and consistent with mouse-models, CD28 blockade via CTLA4-Ig alone does not fully prevent T cell activation (9). The use of CTLA4-Ig as a monotherapy has proved to be insufficient to induce long-term allograft acceptance in several transplant settings (7, 8, 10). Additionally, concerns with its long-term administration have also been raised due to a possible impact on the homeostasis of Tregs (11, 12). Consequently, many investigations have been trying to identify the main factors limiting CTLA4-Ig efficiency and to design clinically relevant complementary regimens to achieve its full therapeutic potential and supporting its successful clinical application.

Multiple studies have demonstrated that events that induce an acute inflammatory response, with the consequent release of proinflammatory cytokines, can prevent the induction of or even break established immunological tolerance promoted via CoB regimens (Figure 1). Examples include the presence of damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) (13–15) or infections (16, 17) at the time of transplantation; ischemia-reperfusion injury (IRI) induced by prolonged graft ischemia (18, 19); and the existence of graft-reactive memory cells induced by previous sensitization events (e.g. previous contacts with donor antigens – blood transfusions, previous transplants, pregnancies – or by heterologous immunity) (20, 21).

Fueled by the shortcomings of CTLA4-Ig, an important area of investigation has been the development of strategies to target additional costimulatory molecules to achieve lasting tolerance. The combined blockade of CD28 and CD154/CD40 signaling pathways was originally proposed as the strategy to achieve lasting graft survival in multiple rodent models of transplantation (9). However, a clinical trial with a humanized antibody to CD154 (the ligand of CD40) was halted due to thromboembolic events (22). Alternative clinically relevant costimulatory pathways (e.g. ICOS-ICOSL, OX40-OX40L, PD1-PDL1, TIGIT-CD155) are also being studied, however, the important advances achieved in this area will not be discussed here, as they have been comprehensively reviewed elsewhere (23).

This review highlights advances in our understanding of the factors (other than costimulatory molecules) that limit the efficacy of CTLA4-Ig-based regimens and discusses recent successes in experimentally designing complementary therapies that unleash CTLA4-Ig full potential, rendering a better control of transplant rejection and supporting their clinical applicability (summarized in Figure 2 and Table 1). Moreover, we introduce novel areas of investigation, and we contextualize how targeting the signaling of inflammatory cytokines via JAK inhibitors could provide a significant advantage.

2. Current landscape of CTLA4-Ig use in combination with other immunosuppressants

The combination of Belatacept with other immunosuppressants has shown benefits in animal models and paved the way to the initiation of clinical trials testing these protocols for clinical applicability (39, 40).

In kidney transplantation, only one calcineurin inhibitor–free regimen, de novo Belatacept in combination with mycophenolate and corticosteroids, is currently FDA approved for use in adult recipients who are seropositive for Epstein–Barr virus. Results have shown this regimen preserves renal function and has a minimal adverse effects profile (41, 42). However, the occurrence of high rates of acute rejection episodes, usually appearing early in the post-transplantation period, has prevented its widespread implementation, and utilization remains below 5% in the US (43). Several studies are evaluating the conversion to Belatacept post-transplant to avoid prolonged CNI exposure while decreasing the risk of acute rejection (44–46). Other investigations suggest that the optimal de novo Belatacept-based regimen may include lymphocyte-depleting induction in combination with an mTOR inhibitor (or Tacrolimus), instead of mycophenolate with or without corticosteroids (47–50).
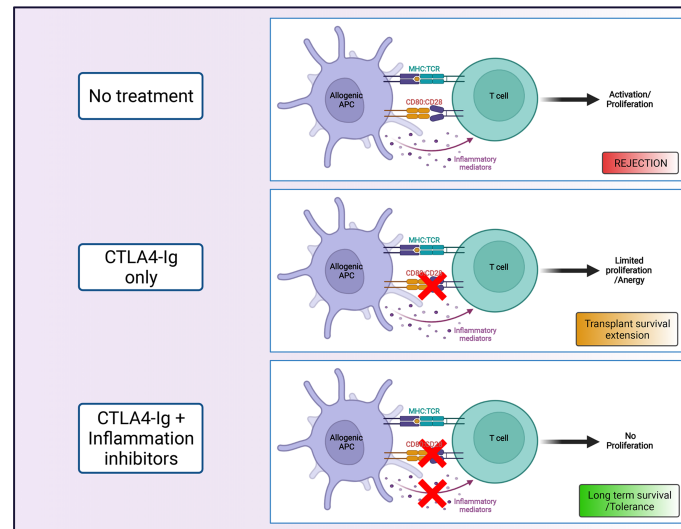
FIGURE 1

Costimulation blockade as strategy to promote transplant survival. The combination of CTLA4-Ig, that blocks interactions between CD28 and CD80/86 (signal 2), with agents that limit the contribution of inflammatory mediators (signal 3) on T cell activation, can promote long term transplant survival (Created with BioRender.com).

The use of Belatacept for liver transplantation in the clinic has provided mixed evidence of efficacy. In a phase II exploratory trial of *de-novo* utilization of Belatacept with MMF, results failed to demonstrate safety or effectiveness, so its use was not recommended (51). Moreover, a follow up study attempting to maintain the same patients on MMF monotherapy after Belatacept withdrawal indicated lack of operational tolerance, as patients experienced graft dysfunction following

the switch (52). Other studies showed some benefits with Belatacept conversion as method for CNI withdrawal (53), or using Belatacept with MMF as bridge to CNI therapy (54).

Belatacept is also a potential alternative to CNI in cardiothoracic transplantation (55). Two retrospective studies in lung and heart transplantation patients that had switched from CNI to Belatacept, as an alternative immunosuppression regimen post-transplantation, reported recovery in renal
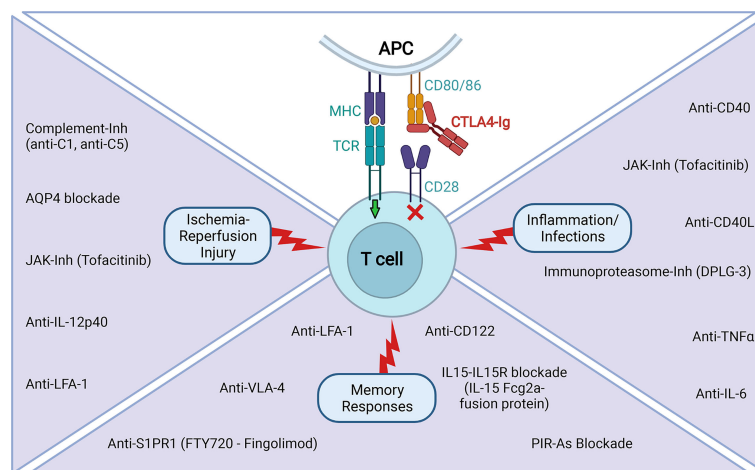
FIGURE 2

Tested experimental strategies to improve CTLA4-Ig efficacy in extending transplant survival. The reported use of agents that limit the contribution of signal 3 on T cell activation in situations of IRI, inflammation and infections, and pre-existence of memory responses that has proven to be efficacious in combination with CTLA4-Ig in extending transplant survival in various experimental settings (Created with BioRender.com).

TABLE 1 List of tested experimental strategies to improve CTLA4-Ig efficacy in extending transplant survival.

Combined therapy	Transplant model	Molecular target
Inflammation inhibitors	Heart, skin (rodents)	Anti-CD40L (9)
	Islets, skin (rodents, NHP)	Anti-CD40 (24, 25)
	Heart (rodents)	Immunoproteasome (DPLG-3) inhibitor (26)
	Heart, islets (rodents)	Anti-IL6 or/and anti-TNFα (27, 28)
	Heart (rodents)	JAK Inhibitors (Tofacitinib) (29)
IRI inhibitors	Heart (rodents)	Complement inhibitors (C1, C5 inhibitors) (19, 30)
	Heart (rodents)	Aquaporin 4 (AQP4) blockade (31)
	Heart (rodents)	Anti LFA-1 (18, 32)
	Heart (rodents)	Anti-IL-12p40 (33)
	Heart (rodents)	JAK Inhibitors (Tofacitinib) (29)
Control memory responses	Islets (rodents)	IL-15 mutant/Fcy2a fusion protein (blockade IL15/IL15R pathway) (34, 35)
	Skin (rodents), kidney (NHP)	Anti-CD122 (IL-2 and IL-15 receptor shared beta-chain) (36)
	Heart (rodents)	Anti-S1PR1 (FTY720 – Fingolimod) (37)
	Skin (rodents)	Anti LFA-1, anti-VLA-4 (20)
	Heart (rodents)	PIR-As blockade (38)

function and an acceptable safety profile (56, 57). More extensive investigations and clinical trials are however needed to obtain the necessary evidence to support this indication. In a rodent study, a short-course CTLA4-Ig-based conditioning regimen, which also included low-dose total body irradiation, bone marrow infusion, tacrolimus and antilymphocyte serum, favored the achievement of mixed chimerism, which correlated with the induction of long-term heart transplant survival and tolerance of secondary donor-specific skin grafts (58).

Experimental data in rodent skin allograft models has also been reported. A combination regimen of CTLA4-Ig with sirolimus or cyclosporine increased effectiveness in an experimental fully MHC-mismatched model of skin transplantation (59). In the same settings, combination with antilymphocyte serum with or without donor-specific bone marrow did not add any benefit to CoB monotherapy. Another report showed that the simple combination of CTLA4-Ig with low-dose anti-murine thymocyte globulin promoted engraftment by promoting Tregs in a context of limited alloreactive effector response (60).

Belatacept-based immunosuppression is also being investigated in the field of Vascularized Composite Allotransplantation (VCA), as extensively discussed in a recent review (61). Supported by the positive outcome of multiple preclinical models, conversion to Beletacept-based immunosuppression (in combination with MMF +/- low dose CNI) has been successfully attempted in a small number of VCA patients. *De novo* use of Belatacept, with MMF, steroids, and tacrolimus, is also being tested, but more data will need to be accrued to properly evaluate the applicability of such a strategy in VCA.

Overall, despite promising benefits, the implementation of Belatacept-based therapies in the clinic remains largely restricted to kidney transplantation. This landscape highlights the need for the identification and optimization of combination strategies with improved therapeutic efficacy that would favor a broader

utilization for management of transplanted patients in multiple clinical settings.

3. Factors that affect CTLA4-Ig efficacy and related targeting approaches

3.1 Inflammation

Multiple preclinical studies have demonstrated that acute inflammatory events can prevent the induction of or even break established tolerance induced *via* CoB regimens (13–17, 62). These events range from surgical injury, infections, and ischemia reperfusion injury that initiate cellular cascades of alarm signals culminating in the production of pro-inflammatory agents. The impact of ischemia reperfusion injury on alloreactivity involves additional unique pathways and will be described in a dedicated sub-section.

Inflammatory cytokines such as type I interferons, IL-1, IFN-γ, IL-6, TNFα provide alternative signals that counteract the inhibition of conventional costimulation. As aforementioned, the combination of CTLA4-Ig with the blockade of CD40 signaling pathway, successfully extended transplant survival in multiple experimental animal models (9). In this context, extension of transplant survival is achieved in part due to the inhibition of secretion of inflammatory cytokines by antigen-presenting cells (APCs) caused by CD40/CD154 blockade (63). Following the interruption of clinical trials due to severe complications (22), multiple groups are currently working on improved versions of the anti-CD40L antibody to avoid the observed side effects (64–66). Alternatively, the blockade of CD40/CD154 pathway with anti-CD40 antibodies (Chi2020, 7E1-G2b), in combination with CTLA4-Ig, showed effects similar to the use of anti-

CD154, inducing long term survival in a non-human primate (NHP) model of islet transplantation (Chi2020), and extending the survival of fully mismatched murine skin transplants (7E1-G2b) (24, 25). However, there are potential limitations in focusing specifically on the CD40 signaling pathway. A recent report suggested that a commonly ignored secondary receptor of CD154, CD11b, is important in favoring the accumulation of graft-infiltrating CD8 T cells after transplantation, independently from the engagement of CD40 (67). This observation explains why blocking CD40 instead of CD154 has been described as less effective for the inhibition of allograft rejection in some scenarios and supports the ongoing efforts to optimize anti-CD154 therapies.

Targeting the immunoproteasome (i-20S), a constitutive isoform of the proteasome highly expressed in T cells, dendritic cells, and B cells, has been explored to control inflammatory diseases like autoimmunity and alloimmunity. This approach would minimize the toxicity associated to the use of non-selective proteasome inhibitors (bortezomib, carfilzomib, ixazomib) which, despite providing good results in inflammatory and autoimmune diseases, can cause general immunosuppression (68). The use of DPLG3 (small molecule inhibitor of the i-20S b5i subunit) for a brief period after transplantation, suppressed cytokine release from blood mononuclear cells and the activation of dendritic cells (DCs) and T cells (26). It also diminished accumulation of effector T cells, promoted expression of exhaustion and coinhibitory markers on T cells, and synergized with CTLA4-Ig to promote long-term acceptance of cardiac allografts across a major histocompatibility barrier.

As above-mentioned, specific inflammatory mediators have been discovered that limit the efficacy of CTLA4-Ig monotherapy regimens. Different reports pointed to IL-1, IL-6, and TNFα as leading proinflammatory cytokines responsible for the anti-tolerogenic effects (69–71). IL-6 can impair the function of Tregs (key to the efficacy of CoB) by promoting proliferation of effector cells (72). The prolongation of transplant survival after CTLA4-Ig administration in recipients lacking this cytokine (IL-6-KO) suggests that the blockade of IL-6, or its signaling pathway, has a synergistic effect with strategies that inhibit T_H1 responses, ultimately promoting long-term allograft survival (70). In line with this observation, treatment with an anti-IL-6 antibody promoted allogenic bone marrow engraftment and prolonged graft survival in an irradiation free murine transplantation model receiving CoB, a result associated with expansion of endogenous Tregs and inhibition of DCs and memory CD8 T cells (27). TNFα also plays an important role in immune regulation (73). It has been found responsible for an increase in T cell allogenic responses (74) and the impairment of peripheral tolerance induction to allogenic pancreatic islets (69). A synergistic role for IL-6 and TNFα acting together to promote T cell alloimmune responses and impairing the ability of Treg

cells to suppress effector T cell alloimmunity has also been described (28). Thus, accumulating evidence suggests the need to combine CoB therapies with inhibition of either the production or the signaling of multiple inflammatory cytokines to achieve a robust modulation of transplant rejection.

The identification of Janus kinases (JAKs), non-receptor tyrosine kinases, as critical components of the signaling pathway of multiple proinflammatory cytokines (e.g. type 1 interferons, IFN-γ, IL-6), made them an attractive drug target to simultaneously inhibit the effect of several inflammatory mediators. Initial efforts lead to the development of JAK inhibitors (JAK-Inh), small molecules that interfere with JAK enzymatic activity (75). JAK-Inh also appeared as a more cost-effective and efficient alternative to biologics to target cytokines, as not all patients respond adequately to biologics-based treatments (76). First generation JAK-Inh were initially tested in transplantation as immunosuppressants and compared with standard of care. In randomized clinical trials in kidney transplantation the use of a fixed dose of tofacitinib, a pan-JAK-Inh currently FDA approved for the treatment of rheumatoid arthritis, psoriatic arthritis, and ulcerative colitis, in combination with mycophenolic acid and corticosteroids, demonstrated a lower incidence of rejection and better renal function than cyclosporine A. However, the safety profile was poor, showing an increased risk of infection and malignancy when compared to CNI-based regimens (77–79). Unfortunately, after these results, the investigation of tofacitinib as a maintenance immunosuppressant in transplantation was halted. Interestingly, revised analysis of the data from these studies and the results of a long-term extension trial, all highlighted the importance of controlling tofacitinib dosing (probably used at too high of a dose in the initial trials) and the need for careful evaluation of the drug combination strategy implemented. It was suggested that close monitoring of the therapeutic dose and targeting reduced exposure over time post-transplant, may improve outcomes and reduce side effects (80). Encouraged by the lessons learned from these reports, the use of tofacitinib as part of a very different combination strategy together with CTLA4-Ig has started to be explored. Using a mouse model of heart transplantation, the combination of CTLA4-Ig with a short period of daily tofacitinib administration revealed a profound synergistic effect that promoted long-term transplant survival (29). The protective effect of the combination of CTLA4-Ig and tofacitinib was associated with inhibition of the maturation of APCs, inhibition of the differentiation of effector T cells, and promotion of intra-graft accumulation of Tregs. This study demonstrated the powerful synergism between CTLA4-Ig and JAKs-inhibition, and suggests their combined use is a promising strategy for improved management of transplanted patients that should be further investigated.

3.2 Ischemia reperfusion injury

Ischemia/reperfusion injury (IRI) is a universal consequence of solid organ transplantation, initiated after interruption of blood supply following organ procurement. In renal transplantation it is currently one of the most prominent causes of delayed graft function (especially for deceased donors), in part due to the use of new allocation systems and novel approaches that have introduced an extension in organ preservation time (81, 82). The negative impact of IRI on cells and tissues arise from the complex network of events including oxygen and nutrient deprivation, disruption of cellular homeostasis, with a switch from aerobic to anaerobic metabolism and resulting in cellular acidification, accumulation of reactive oxygen species and inflammation. An increased release of proinflammatory mediators, cytokines, chemokines, and expression of adhesion molecules are induced by IRI and contribute to ischemic tissue damage resulting in the release of DAMPs (83, 84). The combination of these effects promotes, directly or indirectly, enhanced anti-donor cellular and humoral responses. Prolonged cold ischemia storage is considered an independent risk factor for poor transplant outcome, having been associated with a higher incidence of delayed graft function, acute and chronic rejection (85). It is also considered one of the leading causes of failure of prolongation of transplant survival by CTLA4-Ig due to its promotion of "costimulation independent" activation of anti-donor responses (18, 19). Different approaches are being investigated to minimize the effects of IRI.

Several studies have revealed that many of the pathological effects of IRI are complement dependent (86). Targeting the complement cascade, which is an important component of the innate immune system, has minimized graft injury initiated by donor reactive antibodies and limited vascular allograft rejection in sensitized recipients (87, 88). Building on this understanding (86), and that alternative complement pathway components contribute to T cell activation and differentiation, inhibition of complement activity has been investigated as an immunomodulatory strategy in transplant settings (89, 90). The inhibition of C5 limited graft injury in human kidney transplant recipients (88). Targeting C5 also had synergistic effect with CTLA4-Ig prolonging survival of murine heart allografts subjected to IRI (30). Mechanistically, the use of anti-C5 mAb prevented the formation of C5a and C5b, resulting in limited induction of T_H1 alloreactive cells and inhibition of primary responses to donor antigens (30). The same group identified an important role for the mannose-binding lectin (MBL) complement pathway (but not the alternative pathway), in the deleterious effect of IRI. CTLA4-Ig treatment of $C3^{-/-}$ recipients of ischemic heart allografts, as well as the use of $mbl1^{-/-}mbl2^{-/-}$ transplant recipients, prolonged survival compared to wild type recipient mice. This prolongation was associated with profound inhibition of the

production of pro-inflammatory cytokines and a limited presence of intra-graft alloreactive activated T cells. Importantly, this group showed a benefit with the use of the FDA approved complement inhibitor C1-INH, which targets the MBL pathway, in combination with CoB. In a murine heart transplantation model, C1-INH was particularly effective when administered within the first 24h post-transplant of ischemic allografts, supporting the need for further studies to test the clinical applicability of this regimen (19).

Promising results were also obtained in experimental transplantation when targeting the Aquaporin 4 (AQP4) signaling pathway. Aquaporins are a family of water channels that facilitate homeostasis but are also involved in modulation of tissue injury and inflammation. AQP4 deficiency results in reduced myocardial tissue damage during infarct and IRI (91). A recent report in a murine model showed encouraging results for the control of IRI in transplantation when AQP4 was blocked during donor allograft collection and storage and short time after transplantation (31). The combined administration of an AQP4 inhibitor and CTLA4-Ig synergistically prolonged the survival of heart allografts. *In vitro* observations of a reduced T cell proliferation and cytokine production following AQP4 blockade, suggest that these effects could help limit the anti-graft response post-ischemia and hence favoring transplant survival (31).

IRI is responsible for the increase in the early infiltration of innate and adaptive leucocytes into the allografts. Neutrophils and macrophages quickly migrate to the ischemic area sensing DAMPs, become activated and lead to a release of chemokines and cytokines (92). Macrophages increase their processing and presentation of allo-antigens, contributing to adaptive immune activation, enhancing the effector functions displayed by early infiltrating memory CD8 T cells, and favoring transplant rejection (18). The simple prevention of early migration of donor specific memory CD8 T cells to the allograft by the administration of anti- leukocyte function associated antigen-1 (LFA-1), an integrin involved in adhesion, activation and trafficking of leukocytes, favored the survival of the transplant (18, 32). In this context, it is plausible to consider an involvement of the recently described "virtual memory" T cells, a population of memory CD4 T cells displaying a T_H1-like phenotype that is generated at the steady state from naïve T cells, in the absence of foreign antigen recognition, and that can be reactivated in a TCR-independent way by IL-12. This population is considered a contributor to enhanced responses in autoimmune and inflammatory diseases (93, 94). With prolonged cold ischemia, endogenous memory CD4 T cells stimulate graft-infiltrating dendritic cells to produce homodimers of IL-12-p40 (IL-12 subunit) that in turn activate anti-graft memory CD8 T cells (33). This process contributes to CTLA4-Ig-resistant allograft rejection observed in a murine model of heart transplantation. The combined therapy of CTLA4-Ig with anti-p40 antibodies proved to be efficacious at

extending transplant survival in the mentioned IRI conditions (33), suggesting the possible use of this strategy to counter CTLA4-Ig-resistant allograft rejection mediated by memory CD8 T cells.

The use of JAK-Inh, already mentioned in the previous section, has also proved to be beneficial to minimize the inflammation and proinflammatory cytokine release characteristic of IRI in a mouse model. Combination of short course of tofacitinib with CTLA4-Ig was able to extend survival of heart allografts subjected to 4h cold ischemia, settings where CTLA4-Ig monotherapy is unable to delay graft rejection. Long-term survival achieved with this combined therapy was associated to a decrease in effector T cell allo-response, and correlated with *in vitro* observations of a full control of T cell proliferation when T cells were stimulated in the presence of both inhibitors, even if exposed to proinflammatory conditions (29). This report highlights the versatility of combining CTLA4-Ig with JAKs-inhibition for improving immunoregulation while limiting the negative effect of inflammatory mediators and indicate the need for additional studies to prove its clinical potential for improving management of transplanted patients.

3.3 Pre-existing memory alloreactivity (sensitization)

One of the biggest barriers to achieving effective modulation of rejection (and possibly allograft tolerance) is the presence of immunological memory toward donor antigens in the recipient before transplantation. Memory cells can develop due to previous sensitization events (e.g. previous contacts with donor antigens through blood transfusions, previous transplants, pregnancies, and by development of heterologous immunity) (20, 21, 95) or *via* homeostatic proliferation while recipients are under immunosuppression (96). Memory responses are faster and more robust, in most cases translating into rejection being more resistant to pharmacologic immunosuppression (97–99). The difficulties presented by pre-existing anti-donor memory apply to CoB regimens too. Memory CD4 and CD8 T cells are less dependent on costimulation for activation, and they can mount a quick allo-response, promote inflammation, and attack and destroy the transplant despite application of CoB treatment. Combination therapies aiming to overcome CTLA4-Ig resistant rejection mediated by memory lymphocytes are being actively investigated.

In humans, different reports indicated that pre-transplant accumulation of different T cell memory subsets are associated with increased risk of CoB resistant rejection. These subsets encompass: a CD28+CD4+ effector memory T cells (100), antigen-experienced CD57+PD1-CD4 T cells (101–103) and a subset of memory CD8 T cells that lacks expression of CD28 (104). IL-15 is a powerful T cell growth factor with particular importance for the maintenance and proliferation of memory

CD8 T cells (105, 106). The CD28-CD8 T cell subset, in both non-human primates and humans, relies on cytokines like IL-15 and IL-2 for activation. CoB cannot act to directly decrease IL-15 expression because epithelial and endothelial cells, and macrophages, not T cells, are the primary cellular sources of this cytokine (107). IL-15 was found to be responsible for the induction of CTLA4-Ig resistant proliferation of alloreactive memory CD8 T cells from renal transplant patients (104). In settings where CoB-resistant rejection could be mediated by memory CD8 T cells, targeting IL-15/IL-15R pathway in combination with CTLA4-Ig represents a potent strategy for the induction of transplant tolerance. The use of an IL-15 antagonist, IL-15 mutant/Fcγ2a (a fusion protein that specifically binds to IL-15Rα, but not to the common γ-chain, shared with other cytokines like IL-2) in combination with CTLA4-Ig, favored the achievement of transplant tolerance in a rodent semi-allogenic islet transplantation system (108). When tested in a fully MHC-mismatched scenario, the combined therapy also extended allograft survival further than CTLA4-Ig alone (34, 35). Additional studies targeting CD122 (IL-15 receptor-β-chain, shared with IL-2) also showed promising results with a synergistic protective effect when combined with CTLA4-Ig (36). The treatment abrogated both primary and memory CD8 T cell responses to transplanted tissues, in mice and non-human primate models. Mechanistic studies dissecting the effect of the anti-CD122 blocking antibody supported a role for IL-15 in memory T cell activation, while the prevention of primary allo-specific responses was more likely due to the blockade of IL-2 signaling (36).

The blockade of migration of memory cells to the graft has also been investigated in combination with CTLA4-Ig. A modification or restriction of lymphocyte homing receptors is a proposed strategy to promote transplant survival. The sphingosine 1-phosphate receptor-1 (S1PR1) functional antagonist FTY720 (FDA approved as Fingolimod), inhibits lymphocyte egress from thymus and lymph nodes (109) and more recently has been described to also hinder DC migration to lymph nodes and their secretion of IL-12 and IL-23 (110). In an experimental murine model of BALB/c.2W.OVA donor heart transplantation into pre-sensitized recipients, the combination of CTLA4-Ig with FTY720 limited anti-donor IFN-γ responses (already achieved with CTLA4-Ig monotherapy), inhibited alloantibody production, and restrained T cell recruitment to the graft, with a consequent extension of transplant survival (37). The levels of donor-reactive effector memory T cells after treatment were lower than pre-transplant, and the authors speculated that the combined therapy might also potentially serve as a T-cell desensitizing protocol (18, 32). Another approach to interfere with memory T cells migration is the use of integrin antagonists. In a skin allograft model where traceable OVA specific CD8 T cells responded to OVA-expressing donor skin, the resistance of memory T cells to CoB consisting of CTLA4-Ig and anti-CD40L, was abrogated when this regimen

was coupled with either anti-VLA-4 or anti-LFA-1 (20). Mechanistic studies revealed that in the presence of CoB, anti-VLA-4 impaired T cell trafficking to the graft but not memory T cell recall effector function, whereas anti-LFA-1 attenuated both trafficking and memory recall effector function. As antagonists against these integrins are already clinically approved (anti-LFA-1, Efalizumab, was approved in 2003, however later suspended in 2009 due to several patients developing progressive multifocal leukoencephalopathy) (111, 112), these findings may have significant translational potential for future clinical transplant trials to minimize CoB resistance associated to the presence of memory CD8 T cells (20).

Initially thought to be confined to T and B lymphocytes, it is now evident that innate myeloid cells can also acquire temporary features of immunological memory, contributing to the amplification of the effects of inflammation and infections after an initial insult (113). This phenomenon defined as "trained immunity" is orchestrated by epigenetic changes (and not by permanent genetic mutations or reprogramming) and confers myeloid, NK cells, and innate lymphoid cells an increased responsiveness to secondary stimuli recognized through pattern recognition receptors (113). In the setting of transplantation, innate myeloid cells such as monocytes and macrophages are able to retain a temporary memory to prior challenges through MHC-I receptors called PIR-As (A-type paired immunoglobulin-like receptors) (38). Ly6C^{hi} monocyte/dendritic cells and macrophages activated in a primary allo-response, mount a greater inflammatory reaction after a second challenge with the same non-self MHC complex, contributing to accelerated transplant rejection. The contribution of innate immunity to transplant rejection has been confirmed in murine models of kidney and heart transplantation with studies using PIR-A blocking antibodies or Pira$^{-/-}$ recipients, showing attenuated responses to donor antigens. The combination of PIR-As blockade with CTLA4-Ig treatment resulted in a synergistic effect, preventing both acute and chronic rejection in a murine model of heart transplantation (38). These recent results indicate that innate memory is another important player in counteracting the therapeutic efficacy of CTLA4-Ig and they suggest a new line of investigation for the development of intervention strategies to improve transplant outcomes.

4. Future outlook

4.1 Belatacept and Tregs

The role of Tregs in the induction and maintenance of tolerance is well-recognized and there are major ongoing efforts to realize Treg-based clinical applications to promote long-term organ allograft survival (114–116). In addition to directly controlling proliferation of T and B cells, Treg also

suppress immune responses and decrease inflammation by limiting the maturation of DCs – ultimately resulting in a more tolerogenic phenotype of these cells, characterized by decreased secretion of proinflammatory mediators and reduced expression of costimulatory molecules (117, 118). Encouraged by positive safety results obtained in phase I trials (119, 120), multiple clinical trials are underway worldwide to test the efficacy of Treg adoptive cell therapy to improve the management of transplanted patients (121). Importantly though, animal models clearly indicate that Tregs are not capable of inducing transplant tolerance when used as single agent (122, 123). In this regard, the possibility of combining CTLA4-Ig with Tregs in transplantation is appealing, but also controversial. As CD28 is required for Treg generation and CTLA4 is essential for Treg function, blockade of the interaction of both molecules with their ligands CD80 and CD86 on APCs by CTLA4-Ig may be detrimental to Treg survival and function. This potential issue is exemplified by the observation that CTLA4-Ig administration accelerated rejection in a single MHC class II-mismatched mouse model of heart transplantation, where the naturally occurring long-term allograft survival is dependent on Tregs (11). Similarly, in another model of single MHC class II-mismatched skin transplantation, where the expansion of endogenous Tregs extends graft survival, CTLA4-Ig reduced Treg-dependent immunomodulation and restored T$_H$1 alloreactivity (12). More recent studies suggest, however, that the counterproductive effects of CD28 blockade on Treg can be avoided by refinement of the dose and timing of CTLA4-Ig administration (124, 125). For example, the adoptive transfer of recipient Tregs obviates the need for cytoreductive conditioning (i.e. irradiation or cytotoxic drugs) in a fully allogeneic bone marrow transplantation model when given together with rapamycin and CoB (anti-CD40L and CTLA4-Ig). This regimen induced durable mixed chimerism and tolerance to skin and heart allografts (126, 127), and its clinical applicability is currently being assessed in an ongoing clinical trial (128). In addition to therapies that involve the transfer of ex-vivo expanded Tregs (polyclonal or Ag-specific using TCR-gene transfer and chimeric antigen receptor technology) to increase the Treg pool (129, 130), other approaches that aim to directly expand Treg *in vivo* are being investigated. These approaches are based on the administration of IL-2 (essential cytokine for Treg expansion), in a concentration or form that is biased for a more selective Treg engagement. Examples are the use of IL-2 mutants (muteins) (131, 132) and IL-2/Anti-IL-2 immuno-complexes (133, 134), all of which are designed to increase cytokine half-life and to skew binding toward Treg cells (expressing the high affinity IL-2 receptor subunit CD25). The combination of these therapies with CoB is a promising strategy to circumvent the possible deleterious effect of CTLA4-Ig and maximize its therapeutic efficacy.

4.2 Metabolic inhibitors

The growing field of immunometabolism has shown that metabolic reactions are not only used for the cells to generate energy to perform their functions, but they are also a way to control immunity and inflammation (135–137). The discovery that T cells have different metabolic requirements depending on their activation status (proliferation, differentiation, effector function) or cell subtype, has opened a new avenue of investigation to learn how to control cell responses by targeting their metabolism. Initial studies in the oncology field have shown that activated T cells markedly upregulate glycolysis even in the presence of oxygen to satisfy the energetic demand of this process, while naïve and Treg cells rely on more conventional processes such as oxidative phosphorylation and fatty acid oxidation (138). Studies in a rodent transplant model demonstrated the immunosuppressive effect of continuous anti-metabolic therapy targeting glycolysis and glutamine metabolism (via administration of the glucose analog 2-Deoxy-d-Glucose, the glutamine analog 6-diazo-5-oxo-L-norleucine, and metformin), resulting in extension of allograft survival through the inhibition of effector cells and the induction of Treg (139). The need for continuous treatment, however, indicated that this anti-metabolic strategy did not promote the actuation of tolerogenic mechanisms capable of sustaining transplant tolerance. However, when metabolic inhibition was paired with CTLA4-Ig, this led to enhanced skin allograft survival and promoted long-term heart transplants acceptance in the absence of maintenance treatment (140). Further investigations of the metabolic demands of immune cells during rejection, together with the identification of more selective inhibitors, has the potential to define a novel therapeutic strategy that can safely and effectively synergize with CoB in the induction of transplant tolerance.

4.3 The application of next generation JAK-Inh

First generation JAK-Inh have been under investigation as therapeutics to control the deleterious effect of proinflammatory mediators in inflammatory and autoimmune diseases, as well as transplantation. Following positive results of the REACH-2 clinical trial in graft-versus-host disease (GVHD), Ruxolitinib, a JAK1/2 inhibitor, obtained FDA approval, and it is now considered the gold standard in glucocorticoid-refractory acute GVHD treatment (141). As aforementioned, a clinical trial of tofacitinib as immunosuppressant in kidney transplantation showed a lower incidence of rejection and better renal function when compared to a cyclosporine-based regimen, but the poor safety profile observed at the doses tested (with a probable contribution by the combination with the anti-

metabolite agent MMF) halted its further clinical investigation (77–79). Additional analysis of data gathered from clinical trials informed that with the proper dosing and careful evaluation of the drug combination employed, the utilization of this inhibitor could provide beneficial results in the transplant field (80). In fact, as discussed in a previous section, the experimental use of a short-course tofacitinib treatment in combination with CTLA4-Ig, demonstrated a synergistic effect extending survival of heart allografts (29). In concordance with other results (142, 143), exposure of DCs to tofacitinib not only reduced the upregulation of costimulatory molecules (by interfering with the JAK/STAT signaling pathway intrinsic to maturation), but it also limited the secretion of factors, like IL-1 and TNFα, that are involved in CoB-resistant transplant rejection (29). However, the impact of JAK-Inh on the homeostasis and function of Tregs needs to be also considered. Long-term administration of JAK-Inh has been associated with a decrease in Treg abundance in multiple settings (144–146). However, other reports indicate that a shorter course does not have a negative impact on this population and that in all cases, the suppressive function of the remaining Treg population is unaffected by JAK-Inh (29, 147, 148). These results clearly suggest the need to further investigate (experimentally and clinically) the utilization of JAK-Inh for the control of multiple inflammatory responses and to support the efficacy of CoB regimens in organ transplantation.

First generation JAK-Inh block all three JAKs and consequently inhibit the action of a number of cytokines on multiple cell types, unfortunately causing significant side effects with their prolonged use. Their promising therapeutic effects sparked great interest in the generation of more selective JAK-Inh, aiming to maintain efficacy while reducing adverse effects (especially those resulting from JAK2 inhibition). Current studies are investigating second-generation JAK-Inh for GVHD (149), several of which have already been FDA approved for the treatment of autoimmune diseases (E.g. Baricitinib, JAK1/2-inh, for RA (150); Upadacitinib, JAK1-Inh, for RA (151); and the not FDA approved Filgotinib, JAK1-Inh (152)) (153). These inhibitors have higher specificity and could present themselves as improved alternative to ruxolitinib or tofacitinib to pair with CTLA4-Ig for a more selective regulation of inflammation (154). From the overview presented herein of the factors that negatively affect CTLA4-Ig efficacy, it is noteworthy that most of the negative effect is directly or indirectly related to inflammatory or homeostatic cytokines, potential targets of modulation by JAK-Inh. It is reasonable to speculate that the use of JAK-Inh could become a valid alternative to more expensive biologics or other aforementioned therapies to maximize CTLA4-Ig efficacy in the development of therapies that will provide safe and effective management of transplanted patients in multiple clinical settings.

In September 2021, the FDA required an update on the prescribing information (a black box warning) for certain JAK-

Inh in the treatment of chronic inflammatory conditions (155). This update came after revision of safety profile studies that determined an increased risk of serious side effects (heart attack, stroke, blood clot, cancer, and death) after continuous and prolonged JAK-Inh use. Considering this new regulation, the additional optimization of administration protocols appears paramount to improve their safety profile and expand their use to other inflammatory disorders and transplantation. The data in rodent models suggesting that the combination of CTLA4-Ig with a transient (rather than continuous/life-long) administration of a JAK-Inh enables proper control of transplant rejection indicates that alternative administration regimens are feasible and effective (29). Implementation of localized delivery systems is also an interesting strategy proven to reduce toxicity deriving from systemic drug exposure, allowing the design of safer strategies of management of transplanted patients. The use of biomimetic nanoparticle platforms and hydrogels are examples of these approaches (156–159). In this regard, a recent study looked at the possibility of limiting the systemic exposure to tofacitinib while maintaining synergism with CTLA4-Ig. Using a single administration of a novel injectable peptide-based hydrogel containing crystals of tofacitinib, the authors demonstrated in a rodent model that delivery of tofacitinib localized exclusively around the transplant preserved synergism with CTLA4-Ig in promoting long term graft survival (160).

5. Conclusions

The limited improvement in the long term management of transplanted patients of the past two decades calls for new and more effective treatment strategies (81). As summarized in this review, research of the past few years revealed novel understanding of the mechanisms of activation of the immune system that challenge old paradigms. These new observations highlight the important, and often unappreciated, role of inflammatory events in limiting the capacity to effectively control transplant rejection. There is then an exciting opportunity for targeting inflammatory perturbations in combination strategies, with realistic translational potential, that will provide better control of alloreactivity. Implementation of biologics, JAK-Inh, or other inhibitors of inflammation could represent the missing piece of this very important puzzle. However, we still need to fully understand the subtle connections to both beneficial and detrimental effects in the utilization of these agents. This notwithstanding, great optimism accompanies the ongoing effort to validate many of these new experimental protocols in large animal models as well as in testing their clinical scalability and efficacy in human transplantation.

Author contributions

MI and GR drafted and reviewed the manuscript. DB and CL reviewed the manuscript and provided insightful feedback. All authors contributed to the article and approved the submitted version.

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Conflict of interest

GR is an inventor in pending patent applications on the localized delivery of small molecule inhibitors (including JAK-Inh). DCB receives research support from Allovir, Amplyx, CareDx, and Natera, he is consultant for CareDx, Hansa, Medeor, Sanofi, and Veloxis and receives honoraria from CareDx, Sanofi, and Veloxis. CPL is on the Scientific Advisory Board of CareDx and Eledon and receives clinical trial support from Bristol Myers Squibb.

The remaining author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Case report: Refractory intestinal Behçet's syndrome successfully treated with tofacitinib: A report of four cases

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Behçet's syndrome (BS) is a chronic form of relapsing multisystem vasculitis, characterized by recurrent oral and genital ulcers. Intestinal BS is a special type of BS. Volcano-shaped ulcers in the ileocecum are a typical finding of intestinal BS, and punched-out ulcers can be observed in the intestine or esophagus. At present, there is no recognized radical treatment for intestinal BS. Glucocorticoids and immunosuppressants are currently the main drugs used to improve the condition. Although it has been reported that monoclonal anti-TNF antibodies may be effective for some refractory intestinal BS, further randomized, prospective trials are necessary to confirm these findings. Some patients are restricted from using biological agents because of serious allergic reactions of drugs, inconvenient drug injections or the impact of the novel coronavirus epidemic. If endoscopic remission (endoscopic healing) is not achieved for a prolonged period of time, serious complications, such as perforation, fistula formation, and gastrointestinal bleeding can be induced. Therefore, it is necessary to develop new treatment methods for controlling disease progression. We reviewed the relevant literature, combined with the analysis of the correlation between the pathogenesis of BS and the mechanism of Janus kinase (JAK) inhibition, and considered that tofacitinib (TOF) may be effective for managing refractory intestinal BS. We report for the first time that four patients with severe refractory intestinal BS were successfully treated with TOF. We hope to provide valuable information on JAK inhibitors as potential therapeutic targets for the treatment of severe refractory intestinal BS.

Introduction

Gastrointestinal (GI) involvement is a serious complication of Behçet's syndrome (BS). Early diagnosis of intestinal BS is difficult because of the lack of specificity in the clinical manifestations of the digestive system and the lack of specific autoantibodies for diagnosis. Therefore, when patients with BS have digestive system symptoms such as abdominal pain, diarrhea, bloody stool, and constipation, endoscopy should be performed as soon as possible to confirm the diagnosis of intestinal BS (1). Simultaneously, NSAID ulcers, inflammatory bowel disease, tuberculosis and other infectious diseases should be excluded as potential causes of these symptoms (2). For patients with intestinal BS who have achieved the disappearance of clinical symptoms and normalization of C-reactive protein (CRP) levels, endoscopic remission (endoscopic healing) is the ultimate treatment goal (3). The response of some patients to traditional treatments is not ideal. Failure to achieve endoscopic remission (endoscopic healing) may lead to serious complications that can seriously affect patients' quality of life.

At present, the exact pathogenesis of intestinal BS remains unknown, but it has been found that T cell immune dysfunction, particularly the activation of helper (Th)1/Th17 cells, the weakening of regulatory T cells (Treg), and the overexpression of proinflammatory cytokines, is considered to be the cornerstone of BS (4, 5). Tofacitinib (TOF), a JAK1/3 inhibitor targeting T-cell signal transduction, inhibits the signal transducer and activator of transcription 1 (STAT1), T-bet phosphorylation, and differentiation of Th1 and Th17 cells (6). TOF has been successfully used in some cases of BS with refractory uveitis and vessel/cardiac involvement (7–9).

Here we report, for the first time, four patients with severe refractory intestinal BS who did not respond well to traditional treatment and could not achieve endoscopic remission (endoscopic healing). However, after TOF treatment, the treatment goal was achieved.

Reports

Clinical characteristics and therapeutic interventions for refractory intestinal BS are shown in Table 1. The intestinal ulcers in four patients with intestinal BS before TOF administration are shown in Figure 1. The efficacy of TOF in the treatment of intestinal BS is shown in Figure 2, including changes in the erythrocyte sedimentation rate (ESR), CRP, the disease activity index of intestinal Behçet's disease (DAIBD), and prednisone dosage during TOF treatment.

The procedure followed in this study met ethical standards and was approved by the ethics committee of Yantai Yuhuangding Hospital (2019372). Informed consent was obtained from the patients for all clinical data, information collection, and drug application, as well as for publication of this case report.

Case 1

A 67-year-old man developed recurrent aphthous ulcers in his mouth starting at the age of 30 years and recurrent penile ulcers starting at the age of 59 years. The patient did not undergo any systematic treatment. On December 8, 2018, he was admitted to a local hospital because of repeated right lower abdominal pain for 8 months and aggravation after eating. Laboratory test results were as follows: fecal occult blood test, positive; hemoglobin (HGB), 96 g/L; ESR, 65 mm/h; CRP, 113.3 mg/L; no abnormality was found in infection, tumor, or immunological indexes. Paired test results were positive. Enteroscopy revealed ulcers in the terminal ileum, ileocecal valve, and colon, which were characterized by large, deep, oval ulcer surfaces, and clear boundaries (Figure 1A). The pathological results of the ulcers showed that the mucosa had severe acute and chronic inflammation accompanied by erosion, inflammatory exudation, granulation tissue formation, the

TABLE 1 Clinical characteristics and therapeutic interventions of refractory intestinal Behçet's Syndrome.

Case	Sex	Age (yrs)	Intestinal BS course (mths)	Clinical features	Previous treatment	TOF combined therapies	Present therapies	Follow-up (mths)	Clinical response
1	M	67	8	O,G,GI ulcers (terminal ileum,ilececal valve,colon)	CS,SASP,MTX, THD,IFX	CS,SASP,THD	TOF 5mg daily,SASP	33	success
2	F	20	7	O,G,S,GI ulcers (ileum,terminal ileum)	CS,AZA,SASP, CTX,THD	CS,SASP,THD	TOF 5mg daily,SASP	20	success
3	F	49	8	O,G,arthralgia,GI ulcers (ileocecum)	CS,THD,AZA, SASP,CTX	CS,SASP	TOF 5mg two times a day,SASP	12	success
4	F	31	18	O,G,GI ulcers (ileocecum)	CS,THD,CTX, SASP	CS,SASP,THD	TOF 5mg two times a day, Pred 2.5mg daily, SASP	10	success

AZA, azathioprine; BS, Behçet' Syndrome; CS, corticosteroids; CTX, cyclophosphamide; EN, erythema nodosum; G, genital ulcer; GI, gastrointestinal; IFX, infliximab; MTX, meth-otrexate; O, oral ulcer; SASP, salazosulfapyridine; THD, thalidomide; TOF, tofacitinib.
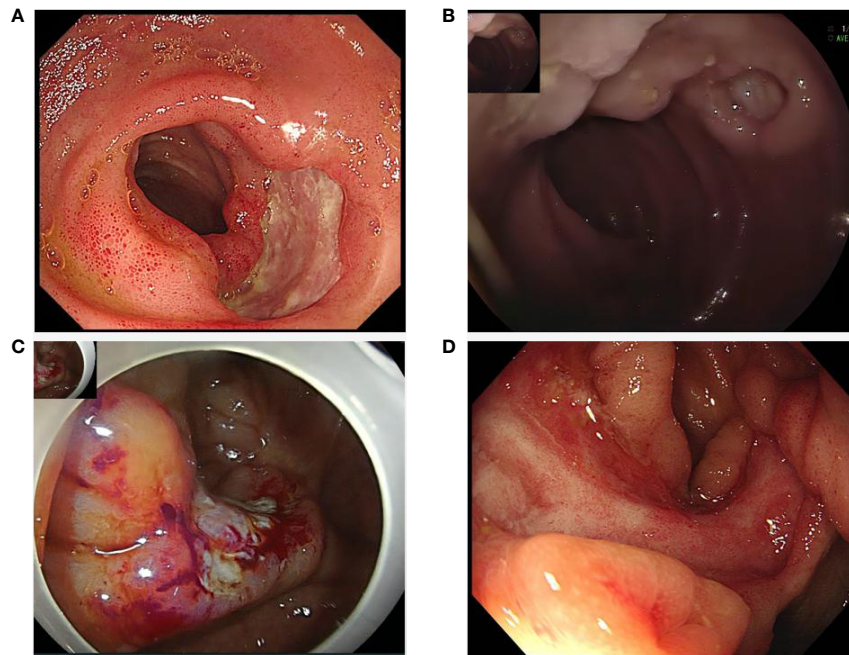
FIGURE 1
Intestinal ulcers in four patients with intestinal Behçet's Syndrome before tofacitinib administration: **(A)** Ileocecal ulcer in case 1; **(B)** Ileum ulcer in case 2; **(C)** Ileocecal ulcer in case 3; **(D)** Ileocecal ulcer in case 4.

infiltration of inflammatory cells in the blood vessel wall and the occlusion of blood vessels. No abnormalities were found on abdominal imaging. The DAIBD score was 90. The patient was treated with prednisone (50 mg PO daily), salazosulfapyridine (SASP, 1.0 g po two times a day), and thalidomide (THD, 50 mg PO per night). His abdominal pain was relieved.

After 4 months, the prednisone dose was gradually decreased to 15 mg daily. However, on April 25, 2019, enteroscopy showed that the large ileocecal ulcer had not healed. The treatment regimen was adjusted to prednisone (60 mg po daily) combined with methotrexate (MTX, 12.5 mg po once a week), SASP, and THD. After 4 months of treatment, the abdominal pain recurred when prednisone was reduced to 15 mg daily. On August 10, 2019, enteroscopy revealed that the ulcer had not healed. The patient was hospitalized in our department on August 20, 2019, for further treatment. Other causes of intestinal ulcers were also excluded from this study. ESR was 72mm/h. CRP was 58mg/L. The DAIBD score was 80. The diagnosis of refractory intestinal BS was clear. The patient developed a generalized itchy rash during infliximab (IFX) treatment. The patient then received TOF (5 mg PO twice a day) and prednisone (20 mg PO daily) combined with SASP and THD. After 4 months of treatment, enteroscopy revealed that the ileocecal ulcer had completely healed. In June 2020, prednisone was gradually reduced to zero. The TOF was reduced to 5 mg/day. To date, the patient's DAIBD score, ESR, and CRP level have been normal (Figure 2).

Case 2

A 20-year-old woman was hospitalized in the digestive department on February 24, 2020, because of abdominal pain and diarrhea for 3 days and bloody stool for 11 hours. The history of the present illness was further investigated. The patient experienced intermittent pain and discomfort in the right lower abdomen without obvious induction 7 months prior and recurrent aphthous ulcer in the mouth and vulva 6 months prior. More than two months ago, nodular erythema recurred on both sides of the shank without induction. In the past two months, the weight dropped by 2.5 kg. Laboratory test results were as follows: fecal occult blood test, positive; HGB, 78 g/L; ESR, 46 mm/h; CRP, 38.5 mg/L. No abnormalities were found in the stool bacterial culture, infection, tumor, or other immunological indices. Paired test results were positive. Abdominal CT revealed a suspected incomplete intestinal obstruction. On February 25, 2020, enteroscopy showed scattered multiple round ulcers of approximately 0.6-2.0 cm that could be seen in the ileum, which were characterized by white moss and obvious edema of the surrounding mucosa (Figure 1B). The biopsy results of the large ulcers showed that the acid-fast bacilli microscopic examination and Mycobacterium tuberculosis rpoB gene examination were negative, and the pathology showed acute and chronic mucosal inflammation.
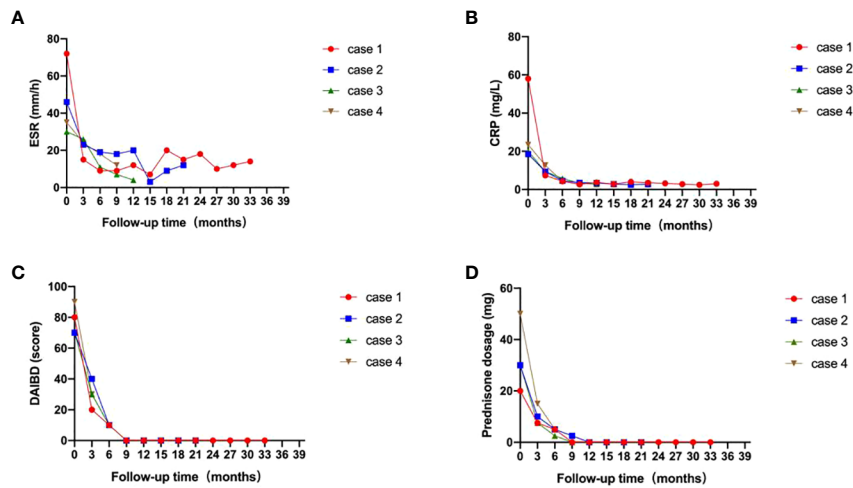
FIGURE 2
Efficacy of tofacitinib for patients with intestinal Behçet's syndrome (red: case 1; blue: case 2; green: case 3; brown: case 4). **(A)** Changes in erythrocyte sedimentation rate (ESR); **(B)** Changes in C-reactive protein (CRP); **(C)** Changes in disease activity index of intestinal Behçet's disease (DAIBD); **(D)** Changes in prednisone dosage, during tofacitinib treatment.

On March 4, 2020, the patient was transferred to the rheumatology department for further treatment. She was diagnosed with severe intestinal BS, with a DAIBD score of 105. The patient was treated with prednisone (30 mg PO daily), azathioprine (ASA, 100 mg PO daily), and SASP. Two weeks later, AZA was stopped due to a decrease in leukocyte levels, which was switched to cyclophosphamide (CTX, 100 mg PO once every other day) after the recovery of leukocyte levels. After 3 months, prednisone was gradually decreased to 10 mg daily. Although the oral ulcer, vulvar ulcer, and nodular erythema significantly improved, the pain persisted in the right lower abdomen. Therefore, the treatment combined with thalidomide was based on the original treatment.

In September 2020, enteroscopy revealed that the ileal ulcer had not healed. Therefore, CTX was discontinued and the patient was switched to TOF (5 mg PO twice a day). Three months later, enteroscopy revealed that the ileal ulcer had healed. In March 2021, the prednisone dose was gradually reduced to zero. In July 2021, TOF was adjusted to 5 mg daily. THD was discontinued in October 2021. To date, the patient's DAIBD score, ESR, and CRP level have been in remission (Figure 2).

Case 3

A 49-year-old woman presented with recurrent aphthous ulcers in the oral cavity and vulva 7 years ago and repeated right lower abdominal pain 6 years ago, accompanied by multiple joint pain but no swelling. After administering THD alone, the frequency of oral and vulvar ulcers decreased significantly, but intermittent abdominal pain persisted. In June 2019, the patient's abdominal pain suddenly worsened, accompanied by diarrhea, with watery stools ranging from 3 to 5 times a day without mucus and pus. The patient was hospitalized on June 26, 2019. In the past year, the patient's weight had dropped by 4 kg. Laboratory test results were as follows: fecal occult blood test, positive; HGB, 114 g/L; ESR, 50 mm/h; CRP, 35.86 mg/L. Examinations were performed to exclude infections, tumors, and other rheumatic diseases. Paired test results were positive. PPD test was negative. Abdominal enhanced CT showed no obvious abnormalities. Enteroscopy revealed an irregular ulcer in 1/2 of the ileocecal cavity, which was characterized by white scar-like changes on the surface, erosion and necrosis, and white moss at the bottom (Figure 1C). Acute and chronic inflammation of the ileocecal region with erosion was observed under pathological examination. The patient's DAIBD score was 90.

The patient was treated with prednisone (40 mg PO daily), AZA (100 mg PO daily), and SASP. After 4 months, prednisone was reduced to 10 mg daily, and the patient still experienced mild discomfort in the right lower abdomen. In October 2019, enteroscopy revealed that the ileocecal ulcer had not healed. The prednisone dosage was adjusted to 30 mg daily. ASA was stopped and switched to CTX (100 mg PO once every other day).

In February 2021, the patient was admitted to the emergency department because of aggravation of abdominal pain. The ESR was 30 mm/h, and the CRP level was 20 mg/L. Further examination was performed to rule out other causes of the ulceration. Enteroscopy revealed the continued presence of the previously noted ileocecal ulcer. The patient's DAIBD score was 80. On February 24, 2021, the patient was treated with

prednisone (30 mg PO daily), TOF (5 mg PO twice daily), and SASP. After two weeks, the patient's abdominal pain gradually subsided, and inflammatory indices gradually decreased. After 5 months, enteroscopy revealed that the ileocecal ulcer had healed. Nine months later, the prednisone dose was gradually reduced to zero. To date, the patient continues to be in remission (Figure 2).

Case 4

A 31-year-old woman developed recurrent aphthous ulcers in her mouth starting at the age of 16 years and recurrent vulvar ulcers starting at the age of 21 years. She had been treated with small doses of prednisone and THD for a short period, but the ulcers recurred after withdrawal. In December 2020, she was admitted to the digestive department of a local hospital because of abdominal pain. Laboratory test results were as follows: ESR, 32 mm/h; CRP, 15 mg/L. Endoscopy revealed an ulcer in the ileocecal region. Pathology showed acute and chronic inflammation accompanied by tissue necrosis, inflammatory cell infiltration, small vessel proliferation, no acid-fast bacilli, and no tumor cells. The patient was diagnosed with intestinal BS.

The patient was treated with prednisone (50 mg/day) and SASP. After 3 months, the prednisone dose was reduced to 20 mg daily. Owing to persistent abdominal pain, she was treated with CTX (100 mg PO once every other day). The patient's abdominal symptoms were not significantly relieved. The patient was admitted to our hospital in August 2021 owing to aggravation of abdominal pain. She had lost 4 kg in the past two months. Laboratory test results were as follows: fecal occult blood test, positive; HGB, 86 g/L; ESR, 35 mm/h; CRP, 23.4 mg/L. Tuberculosis and the tumor were ruled out. Abdominal CT showed thickening and roughness of the ileocecal wall, which was considered an inflammatory change. On August 17, 2021, enteroscopy showed that an ulcer with a size of about 4.0 cm x 1.2 cm in the ring 2/3 cavity was found near the ileocecal valve, which was characterized by white scar in the local mucosa, congestion and edema in the surrounding mucosa (Figure 1D). Pathological examination revealed acute and chronic inflammation of the mucosal tissue. The patient's DAIBD score was 80.

The patient was treated with prednisone (50 mg PO daily), TOF (5 mg PO twice a day), SASP, and THD (discontinuation due to obvious reduction in menstrual volume). After two weeks, the abdominal pain gradually subsided. Subsequently, the prednisone dose was gradually reduced to 2.5 mg daily. In December 2021, enteroscopy revealed that the ileocecal ulcer had healed, and the patient's DAIBD score, ESR, and CRP levels were normal (Figure 2).

Discussion

This paper reports four cases of patients with refractrory intestinal Behçet's syndrome characterised by obstinate peptic ulcers and sustained gastrointestinal symptoms, successfully treated with Tofacitinib. It has been reported that some BS patients without gastrointestinal symptoms can have peptic ulcers on endoscopy (10). Therefore, once patients with BS have gastrointestinal symptoms, gastrointestinal endoscopy should be performed as soon as possible to determine the diagnosis of intestinal BS, which can help identify peptic ulcer early and permit clinicians to intervene in time to reduce the occurrence of complications.

Behçet's syndrome (BS), also known as Behçet's disease (BD), is characterized by a concurrence of innate and adaptive immune disorders and is considered to be an intermediate between the innate (autoinflammation) and adaptive (autoimmunity) immune disease (11, 12). The main manifestations are recurrent oral ulcers, genital ulcers, uveitis, and skin damage, as well as damage to peripheral blood vessels, heart, nervous system, gastrointestinal tract, joints, lungs, and kidney (13, 14). BS is classified as variant vasculitis in the nomenclature of the Chapel Hill Consensus Conference (CHCC) vasculitis revised in 2012 (15).

Intestinal BS is a special form of BS. The incidence of intestinal BS varies from 4% to 38% (16). The age of onset is usually in the range of 15–50 years of age, and the incidence rate is similar between men and women. Among the four patients described in this report, three were young and middle-aged women and one older male. The entire digestive tract can be affected, from the esophagus to the anus, particularly the terminal ileum, ileocecum, and ascending colon (17). The clinical manifestations of gastrointestinal tract involvement also vary, including abdominal pain, abdominal mass, diarrhea, abdominal distension, dysphagia, vomiting, bloody stools, and constipation (18). Severe cases may be complicated by peptic ulcers, bleeding, intestinal perforation, intestinal obstruction, and fistula formation. Due to the lack of specific clinical manifestations and autoantibodies in patients with intestinal BS, the diagnosis is often delayed.

Typically, volcano-shaped ulcers around the ileocecal region are observed in intestinal BS; these are characterized by round or oval shape (number ≤ 5), clear boundaries, diameter greater than 1 cm, and deep wounds. CT findings of the intestinal tract may show thickening of the intestinal wall and peri-intestinal infiltration shadow, and some may show mesenteric vascular congestion, fistula formation, and surrounding adipose tissue turbidity (3), such as in cases 2 and 4.

Pathological manifestations are nonspecific manifestations of acute and chronic inflammation of the intestinal mucosa. Gastrointestinal manifestations usually occur 4.5–6 years after the onset of oral ulcerations. However, intestinal lesions can sometimes precede extra-intestinal manifestations (19). The four cases described in this report all started with oral and/or genital ulcers, and intestinal involvement occurred months or years later. Many BS patients do not pay attention to oral ulcers at the early stage of onset, and then later visited a doctor because of

severe genital ulcers or severe abdominal pain. Therefore, some patients with intestinal BS first visit the digestive department. When a patient has typical volcano-shaped ulcers in the ileocecal region, the doctor needs to carefully ask for the patient's past medical history to ensure accurate diagnosis and treatment.

According to the 2014 international diagnostic criteria for BS (1), all four patients described in this report were diagnosed with BS. The diagnosis of intestinal BS was confirmed using gastrointestinal symptoms and endoscopy. The evaluation of intestinal BS includes clinical symptoms, HGB, ESR, CRP, endoscopy, and DAIBD score, which have guiding significance for the evaluation of treatment effects (20). Endoscopic remission (endoscopic healing) is the most important aim in patients with intestinal BS. All four patients with intestinal BS in this report had a DAIBD score of more than 75 before initial treatment, which was defined as severe intestinal BS. After they were diagnosed with intestinal BS, they were all treated with medium to large doses of glucocorticoids combined with a variety of immunosuppressants; however, the patient's abdominal pain recurred repeatedly, and the intestinal ulcer did not heal for a long time. The patients were thus diagnosed with refractory intestinal BS.

At present, there is no radical cure for intestinal BS. The evidence for effective treatment of intestinal BS mainly depends on retrospective observational data, and there are few controlled clinical studies. The purpose of treatment is to induce and maintain relief of gastrointestinal symptoms, promote mucosal healing, reduce recurrence, and avoid surgical treatment and irreversible intestinal injury. The EULAR guidelines suggest that patients with moderate to severe intestinal BS require glucocorticoids combined with immunosuppressants (2). Patients with refractory intestinal BS generally respond poorly to traditional treatment, and TNF inhibitors (infliximab or adalimumab) can be considered (2, 15, 21, 22). However, owing to serious adverse reactions and inconvenient drug injection or the influence of novel coronavirus, some patients are restricted from using biological agents. Moreover, the persistent failure to achieve endoscopic remission (endoscopic healing) can lead to serious complications. Therefore, it is necessary to develop new treatment methods for controlling disease progression.

At present, there have been some cases of successful treatment of refractory BS eye disease and vessel/cardiac involvement with TOF. Liu et al. reported that six patients with refractory intestinal BS received TOF treatment; the intestinal ulceration healed in one patient and persisted in the other five patients (7). However, three of those patients had very serious complications, such as fistula formation or perforation. The four patients described in this report with refractory intestinal BS (without perforation and fistula formation)

achieved good results after TOF treatment, including relief of clinical symptoms, reduction of inflammatory indicators, gradual healing of intestinal ulcers, and gradual withdrawal of glucocorticoids in some patients. Therefore, we suggest that the early addition of TOF to patients with refractory intestinal BS with persistent nonhealing of intestinal ulcers after active traditional drug treatment may be a better option for those patients who do not have severe complications such as perforation or fistula.

Thus, tofatinib may be a potential treatment for refractory intestinal BS. The Janus kinase/signal translator and activator of transcription (JAK-STAT) signaling pathway and its corresponding cytokines have been implicated in the pathogenesis of BS (5). TOF can inhibit JAK, STAT1, and T-bet phosphorylation, which block the signaling of interleukin (IL)-2, IL-4, IL-6, IL-23, interferon (IFN -γ), and IFN -α, and suppress the differentiation of Th1 and Th17 cells (6). Transcriptome analysis of patients with BS demonstrates that Th17 related genes and type I IFN-inducible genes are upregulated, and that JAK/STAT signaling promotes the activation of Th1/Th17 cytokines (23).

TOF has been approved for the treatment of rheumatoid arthritis (RA), ankylosing spondylitis (AS), psoriatic arthritis (PsA), ulcerative colitis (UC) and other inflammatory diseases (24). These diseases and BS share some common clinical features and genetic variants (24). For example, they share the same genetic background as the JAK/STAT-activated cytokines IL-23R and IL-21R (25). The four patients with refractory intestinal BS described in this report did not respond well to traditional treatment, and one of the patients had adverse reactions after receiving IFX. After receiving TOF treatment, all patients achieved disappearance of clinical symptoms, normalization of CRP levels, and endoscopic remission. Among them, glucocorticoids were gradually discontinued in three patients. The TOF dose was halved in two patients. We suggest that TOF had a definite effect on refractory intestinal BS in these four patients.

In conclusion, considering the key role of abnormal activation of T cells in the pathogenesis of BS, the JAK/STAT signaling pathway may be a potential target for the treatment of patients with refractory intestinal BS. JAK inhibitors are potential treatments for refractory intestinal BS.

This case report has limitation. The number of cases reported in this case report is relatively small, including only 4 patients with refractory intestinal BS. Although TOF is effective in these four patients with refractory intestinal BS, however, we will increase the number of clinical applications in future work, further evaluate the clinical efficacy of TOF in the treatment of refractory intestinal BS, and conduct multi-center research to confirm the efficacy and safety of JAK inhibitors.

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 the ethics committee of Yantai Yuhuangding Hospital (2019372). The patients/participants provided their written informed consent for the publication of any potentially identifiable images or data presented in the article.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Check for updates

Induction of T cell exhaustion by JAK1/3 inhibition in the treatment of alopecia areata

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Alopecia areata (AA) is an autoimmune disease caused by T cell-mediated destruction of the hair follicle (HF). Therefore, approaches that effectively disrupt pathogenic T cell responses are predicted to have therapeutic benefit for AA treatment. T cells rely on the duality of T cell receptor (TCR) and gamma chain (γc) cytokine signaling for their development, activation, and peripheral homeostasis. Ifidancitinib is a potent and selective next-generation JAK1/3 inhibitor predicted to disrupt γc cytokine signaling. We found that Ifidancitinib robustly induced hair regrowth in AA-affected C3H/HeJ mice when fed with Ifidancitinib in chow diets. Skin taken from Ifidancitinib-treated mice showed significantly decreased AA-associated inflammation. $CD44^+CD62L^- CD8^+$ T effector/memory cells, which are associated with the pathogenesis of AA, were significantly decreased in the peripheral lymphoid organs in Ifidancitinib-treated mice. We observed high expression of co-inhibitory receptors PD-1 on effector/memory $CD8^+$ T cells, together with decreased IFN-γ production in Ifidancitinib-treated mice. Furthermore, we found that γc cytokines regulated T cell exhaustion. Taken together, our data indicate that selective induction of T cell exhaustion using a JAK inhibitor may offer a mechanistic explanation for the success of this treatment strategy in the reversal of autoimmune diseases such as AA.

Introduction

Alopecia areata (AA) is an autoimmune disease of the hair follicle (HF) which results in hair loss that ranges in presentation from circular patches on the scalp to total scalp hair loss to full-body hair loss (1–4). The etiology of AA has been shown to involve a combination of genetic predisposition and environmental triggers (2–4). Due to the significant psychosocial burden of AA, affected patients experience enormous psychological and emotional stress (5, 6).

We previously showed that cytotoxic NKG2D$^+$CD8$^+$ T lymphocytes are necessary and sufficient for AA development (7). The pathogenesis of AA is also associated with the overexpression of cytokines, including interferon gamma (IFN-γ) and the common gamma chain (γc) cytokines interleukin (IL)-2, IL-7, and IL-15, which promote the activity of alopecic T lymphocytes in affected skin (3, 4, 7). The IFN-γ and γc cytokines signal through receptors that utilize Janus kinases (JAKs) as their downstream effectors. These signals are subsequently propagated by signal transducers and activators of transcription (STATs) to regulate the expression of associated genes (8). Since JAK-STAT pathways play an essential role in both innate and adaptive immunity, dysregulation of JAK-STAT signaling has been implicated in multiple autoimmune disorders (8, 9). Indeed, inhibition of JAK activity by small molecule inhibitors has demonstrated clinical efficacy in rheumatoid arthritis (RA) and other autoimmune diseases (8–11). Recent progress in treating AA has been demonstrated with JAK inhibitors such as ruxolitinib (a JAK1/2 inhibitor) and tofacitinib (a pan-JAK inhibitor), which both robustly restored hair regrowth in the C3H/HeJ mouse model and in patients with AA (7, 12–14).

Signaling through the six γc cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) requires JAK3 binding to the γc present in the receptor complexes for all six cytokines, as well as JAK1 binding to the other chains within the receptors (5). Through these JAK-receptor interactions, each γc cytokine induces differential activation of STAT pathways to achieve specific effects. Dual JAK1 and JAK3 inhibition can effectively suppress γc cytokine signaling by inhibiting downstream signal transduction (15, 16). Therefore, a selective JAK1/3 inhibitor could provide increased therapeutic efficacy through suppression of AA-associated cytokines (γc cytokines and IFNs) while avoiding unwanted JAK2-mediated side effects (8, 9).

To test this hypothesis, we used Ifidancitinib, a selective next-generation JAK1/3 inhibitor, in the C3H/HeJ mouse model of AA (17). We found that Ifidancitinib inhibited JAK1/3 function *in vitro* and reversed AA in C3H/HeJ mice *in vivo* with reduced pathogenic T cell responses and decreased infiltration of immune cells into the skin. Moreover, the effector/memory T cells exhibited a profile of markers of T cell exhaustion after treatment with Ifidancitinib. We found that blockade of γc cytokine signaling through other JAK1 or JAK3 inhibitors promoted T cell exhaustion. Our results provide rationale for pursuing clinical studies of selective next generation JAK inhibitors to inhibit JAK1 or JAK3 signaling in therapies for patients with AA.

Results

Ifidancitinib inhibited JAK1/3 signaling pathways

Ifidancitinib is a selective next-generation JAK1/3 inhibitor predicted to disrupt γc cytokine and IFN-γ signaling. We characterized the effects of selective inhibition of JAK/STAT signaling in mouse T cells by treatment with Ifidancitinib. We found that treatment of mouse T cells with Ifidancitinib over a range of doses strongly inhibited IL-2-stimulated STAT5 phosphorylation (Figure 1A). Ifidancitinib also exhibited potent inhibitory effects on JAK1/2-mediated IFN-γ signaling, as shown by its suppression of STAT1 phosphorylation upon stimulation with IFN-γ (Figure 1B). These findings demonstrate that Ifidancitinib is a potent JAK1/3 inhibitor.

To directly test the effect of Ifidancitinib-mediated JAK1/3 inhibition on the development of NKG2D$^+$CD8$^+$ T cells *in vitro*, we treated IL-15 stimulated T cells with Ifidancitinib at the start of culture. The presence of NKG2D$^+$CD8$^+$ T cells was quantified after 4 days, and as expected, IL-15 robustly induced the generation of NKG2D$^+$CD8$^+$ T cells (Figure 1C). Compared to vehicle control, however, Ifidancitinib treatment significantly reduced the population of NKG2D$^+$CD8$^+$ T cells in a dose-dependent manner (Figure 1D). Accordingly, the proportions of IFN-γ producing CD8$^+$ T cells were also markedly reduced by Ifidancitinib treatment (Figures 1E, F). These results indicated that Ifidancitinib-mediated JAK inhibition suppressed the generation and differentiation of IFN-γ producing NKG2D$^+$CD8$^+$ T cells *in vitro*.

Ifidancitinib impaired murine T cell proliferation *in vitro* and *in vivo*

C3H/HeJ mice with AA exhibit a striking cutaneous ly0mphadenopathy with increased total cellularity, which may be mediated by a milieu of γc cytokines and other helper cells (7). Therefore, we examined the effect of Ifidancitinib on *in vitro* T cell proliferation driven by IL-2, a known γc cytokine important for T cell proliferation (18). CellTrace Violet-labeled T cells were co-stimulated with anti-CD3 Ab and IL-2 and subsequently treated with either Ifidancitinib or vehicle control. As determined by dye dilution and subsequent flow cytometry analysis, IL-2 strongly promoted T cell proliferation in the vehicle control as expected (Figures 1G, H). In contrast, Ifidancitinib strongly suppressed T cell
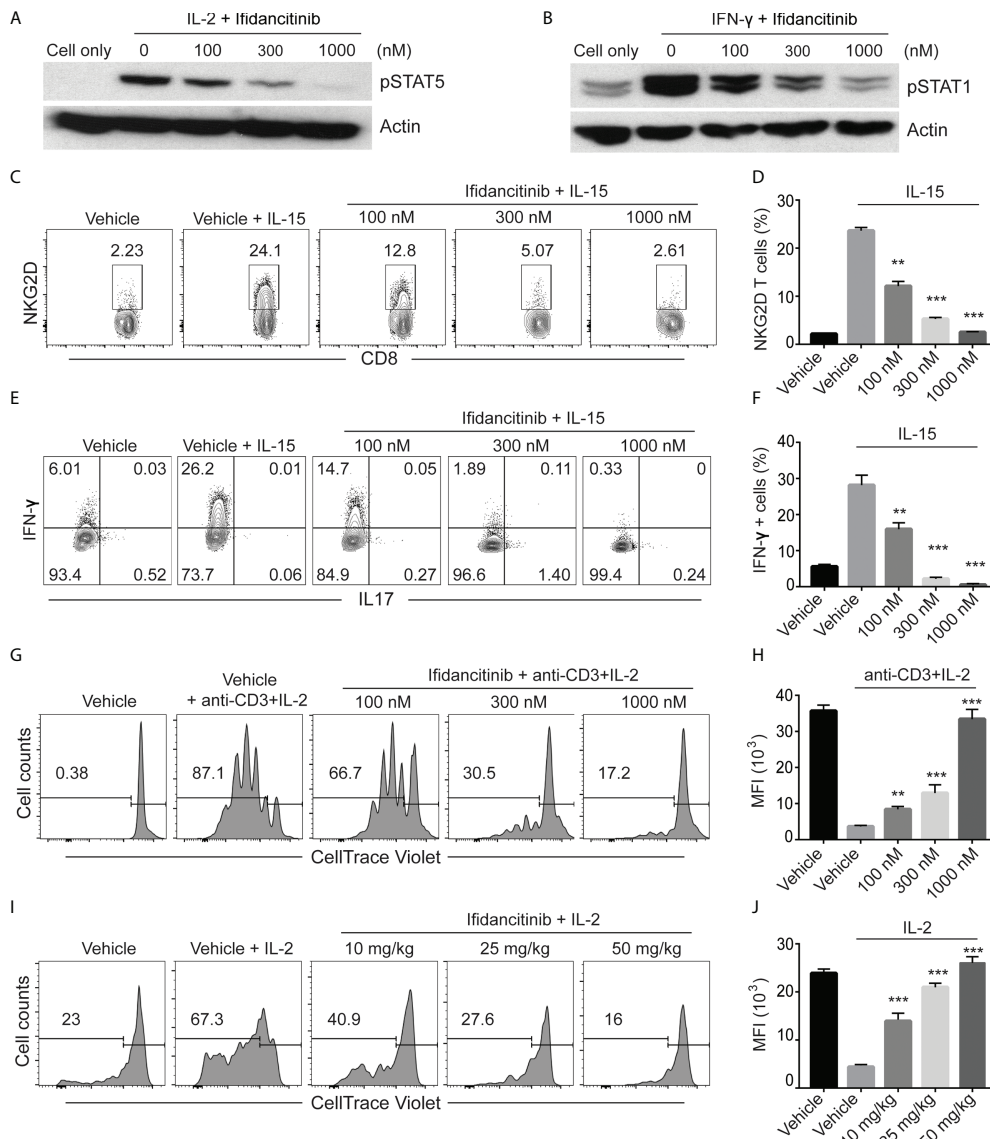
FIGURE 1

Ifidancitinib inhibited γc cytokine signaling. **(A)** and **(B)** *In vitro* activated T cells were pretreated with various dose of Ifidancitinib or vehicle (DMSO) for 1 h and stimulated with rhIL-2 (20 ng/ml) or rmIFN-γ (40 ng/ml) for 15 min, and then cell lysates were subjected to immunoblotting with the indicated Abs. **(C)** to **(F)** CD8 T cells from normal haired C3H/HeJ mice were stimulated with rmIL-15 (50 ng/ml) in the presence of increasing dose of Ifidancitinib or DMSO for 4 (d) On day 5, NKG2D expression was analyzed by flow cytometry **(C)** and **(D)**. In **(E)** and **(F)**, cells were restimulated with cell stimulation cocktail in the presence of plus protein transport inhibitors and analyzed for the intracellular expression of IFN-γ and IL-17. **(G)** and **(H)** CellTrace Violet labeled CD4+ or CD8+ T cells were stimulated with anti-CD3 and IL-2 in the presence of DMSO or increasing dose of Ifidancitinib for 4 (d) Proliferation of the T cells were measured by dilution of CellTrace Violet. **(I)** and **(J)** CellTrace Violet dye stained CD45.1 T cell blasts from B6 Cd45.1 were adoptively transferred to B6 CD45.2 recipients. CD45.2 recipients were then treated with Ifidancitinib and 20µg rhIL-2. Proliferation of the CD45.1 T cells were measured by dilution of CellTrace Violet. **P < 0.01, ***P < 0.001 (one-way ANOVA). The results are representative of two separate experiments.

proliferation in a dose-dependent manner (Figures 1G, H). Next, to test whether Ifidancitinib could block γc cytokine-driven T cell proliferation *in vivo*, we adoptively transferred CellTrace Violet-labeled B6 CD45.1 T cell blasts into B6 CD45.2 recipient mice, which were then treated with IL-2 for two days in order to promote

T cell proliferation (18). Recipient mice were also treated with varying doses of Ifidancitinib over the same time period and the proliferation of transferred T cells in their lymphoid organs was determined after the two days of treatment. As determined by dye dilution, Ifidancitinib inhibited IL-2-driven T cell proliferation in a

dose-dependent manner (Figures 1I, J). Taken together, these results indicated that Ifidancitinib robustly inhibited IL-2-driven mouse T cell proliferation both *in vitro and in vivo*.

Ifidancitinib impaired human T cell function *in vitro*

We next evaluated the effects of Ifidancitinib on human T cells. Purified CD8$^+$ T cells were stimulated with anti-CD3 Ab in the presence of IL-15 to induce NKG2D expression (7). Compared to the control, Ifidancitinib not only robustly inhibited NKG2D expression (Figures S1A, B), but also significantly decreased the proportions of IFN-γ producing CD8$^+$ T cells (Figures S1C, D). Furthermore, Ifidancitinib reduced the proliferative response of T cells in response to anti-CD3 and IL-2 stimulation in a dose-dependent manner (Figures S1E, F). These results demonstrate that pharmacological inhibition of JAK1/3 signaling *via* Ifidancitinib impaired human T cell function *in vitro*.

Treatment with Ifidancitinib prevented the development of AA

Given the striking effect of Ifidancitinib on the suppression of γc cytokine-driven T cell proliferation and function, we next tested the effect of Ifidancitinib on disease development in the C3H/HeJ skin graft model of AA. Upon engraftment of AA affected skin from C3H/HeJ AA donor mice, young recipient C3H/HeJ mice were fed either normal chow or Ifidancitinib-incorporated chow diet (0.5 g/kg) (19). In the control group, all mice developed AA by 8 weeks after engraftment. In contrast, none of Ifidancitinib treated-mice developed AA by 12 weeks after engraftment (Figures 2A, B). Concordant with the prevention of disease, immunofluorescence staining of skin revealed scant CD8$^+$ infiltrates in Ifidancitinib-treated animals, compared to striking infiltration of CD8$^+$ T cells in the skin of control mice (Figure 2C). Ifidancitinib-treated mice also showed significantly reduced staining for MHC class I and II on HF epithelium compared to controls (Figure 2C). Flow cytometry of single cell suspensions of skin samples confirmed reduced expression of MHC class I and II on HF (CD45-EpCAM+CD200+) in Ifidancitinib-treated mice compared to controls (Figure 2D) (20). Furthermore, flow cytometry of single cell suspensions of skin samples further showed that CD45$^+$ inflammatory infiltrates, including CD4$^+$ T cells and CD8$^+$ T cells, were significantly diminished in skin from Ifidancitinib-treated mice compared to controls (Figures 2E, F). In addition, Ifidancitinib significantly reduced the frequencies and total cell numbers of both CD8+NKG2D+ T cells and CD8+CD44+CD62L- effector memory T cells in skin-draining lymph nodes (SDLN) (Figures 2G–I). Taken together, these

results indicate that JAK1/3 inhibition by Ifidancitinib prevented disease onset in the C3H/HeJ skin grafted mice.

Treatment with Ifidancitinib reversed AA in C3H/HeJ mice

To address whether Ifidancitinib effectively reverses disease in AA-affected mice, C3H/HeJ mice that developed AA 8-10 weeks after engraftment were treated with Ifidancitinib-incorporated chow diet (0.5 g/kg) (19). Following 12 weeks of treatment, robust hair regrowth was observed in Ifidancitinib-treated mice, compared to sustained and progressive hair loss in control mice (Figures 3A, B). Histological analyses on skin biopsies taken after treatment showed that Ifidancitinib-treated mice exhibited substantially reduced AA-associated inflammatory infiltrates and inflammatory markers (CD8, MHC class I and II) (Figure 3C). Additional analyses by flow cytometry of single cell suspension of skin samples confirmed that Ifidancitinib significantly reduced inflammatory infiltrates in the skin (Figures 3D, E). Role of tissue-resident memory T cells (T$_{RM}$) in pathogenesis of AA was implicated in patients with AA but remains poorly understood (21, 22). The percentage and total number of CD69$^+$CD103$^+$CD8$^+$ T$_{RM}$ were markedly reduced in the skin of Ifidancitinib-treated mice compared to skin of control mice. Flow cytometry showed that the proportions of IFN-γ producing CD8$^+$ T cells in the skin were also markedly reduced in Ifidancitinib-treated mice (Figures 3D, E).

To gain further insight into the molecular responses to Ifidancitinib, we performed bulk RNA sequencing (RNA-seq) on the skin of treated mice. We found that Ifidancitinib induced dramatic changes in gene expression in treated mice at 10 weeks and shifted gene expression patterns towards the pattern of non-alopecic C3H/HeJ mice (Figure S2A). We showed that the sets of genes selected to comprise our IFN and CTL signatures-Cd8a, Gzmb, Icos and Prf1 for the CTL signature and Cxcl9, Cxcl10, Cxcl11, Mx1, and Stat11 for the IFN signature were significantly decreased in mice responsive to treatment with Ifidancitinib (Figure S2B) (7). In summary, these results indicate that the JAK1/3 inhibitor Ifidancitinib reduced the presence of inflammatory infiltrates and attenuated their function in skin, leading to effective reversal of established disease.

Effects of Ifidancitinib on immune cell populations in peripheral lymphoid organs

We next investigated mechanisms underlying the reduction of infiltrating T cells in the skin during Ifidancitinib treatment. We examined whether treatment affected the number, phenotype, or function of T cells or T cell subsets in
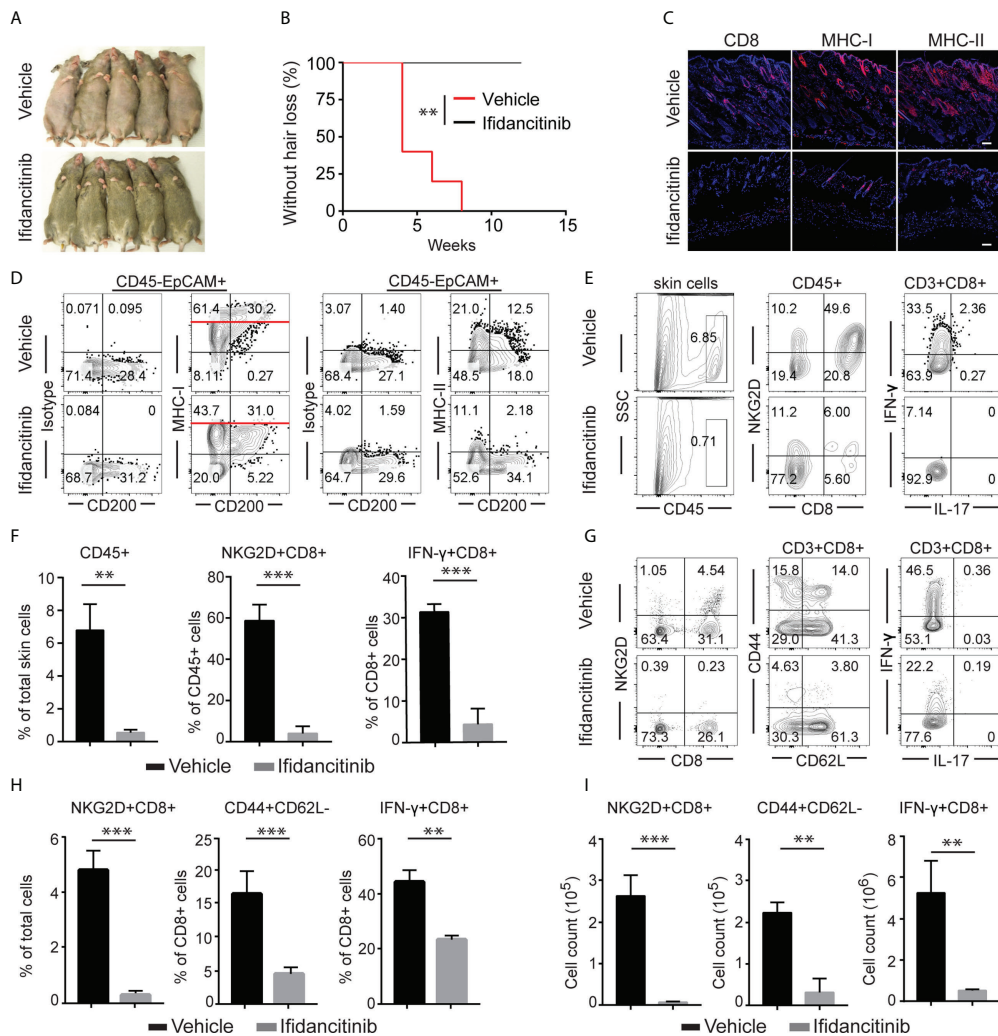
FIGURE 2

Ifidancitinib prevented the onset of alopecia in AA skin grafted C3H/HeJ mice. Mice were given either Ifidancitinib (n=10) or vehicle control (n=10) in chow diets beginning the day of grafting. AA skin grafted C3H/HeJ mice were given either Ifidancitinib or vehicle control in chow diets for 12 wks. **(A)** The onset of alopecia was inhibited by Ifidancitinib. **P < 0.01, log-rank test. **(B)** Time course of onset of AA in control mice and Ifidancitinib treated mice was shown as weeks after grafting. **(C)** Representative immunofluorescence images of skin sections stained with anti-CD8, anti-MHC-I, or anti-MHC-II mAbs. Dashed scale bars represent 100 μm. **(D)** Representative FACS plots of skin cell suspension showed the expression of MHC class I and II on HF (gated on CD45-EpCAM+CD200+). **(E)** and **(F)** Representative FACS plots of cell suspension of mouse skin and the frequencies of infiltrating CD45^{+} leukocytes and IFN-γ-producing CD8^{+}NKG2D^{+} T cell in skin of Ifidancitinib treated mice and control mice. **P < 0.01, ***P < 0.001 (Unpaired Student t test). **(G)** to **(I)** Representative FACS plots of cell suspension of mouse SDLNs **(G)**,the frequencies **(H)** and total cell numbers **(I)** of indicated T cell subsets. **P < 0.01, ***P < 0.001 (Unpaired Student t test).

peripheral lymphoid organs. We found that AA mice treated with Ifidancitinib had a significantly reduced absolute number of total CD3 T cells, CD4 and CD8 T cells in the SDLNs compared with controls (Figure S3). CD8^{+}NKG2D^{+} T cells and CD8^{+}CD44^{+}CD62L^{-} effector memory T cells in SDLNs, which are associated with AA pathogenesis, were diminished in terms of both absolute number and percentage of total T cells (Figure S3). These results indicate that Ifidancitinib treatment resulted in a profound reversal of T cell activation and proliferation in peripheral lymphoid tissues.

Treatment with Ifidancitinib induced effector T cell exhaustion

In the setting of chronic viral infection, which results in the sustained disruption of the γc cytokine network, effector T cells might undergo exhaustion (23). Exhausted T cells display high levels of PD-1 and reduced production of effector cytokines, such as IFN-γ (23, 24). Previous studies showed that Jak3 knockout T cells have impaired γc cytokine signaling and show increased expression of PD-1 and LAG-3 (25). Due to its
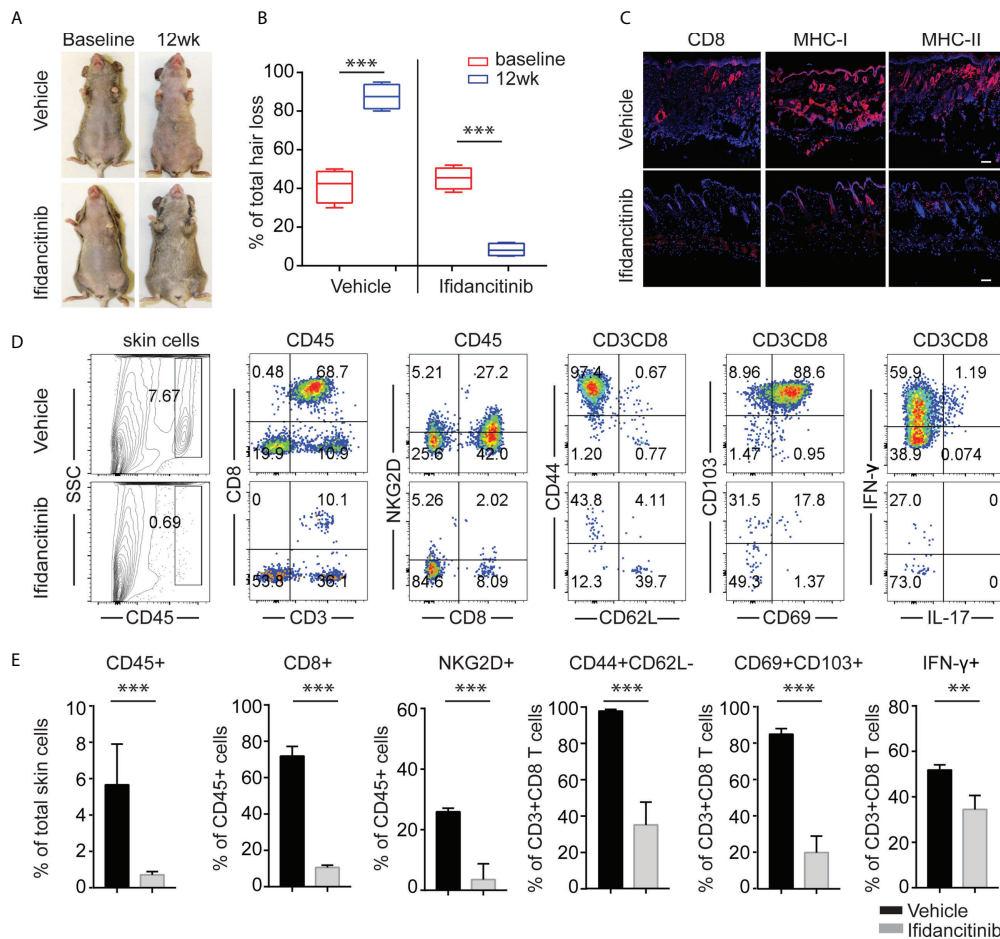
FIGURE 3
Reversal of established AA with Ifidancitinib treatment. C3H/HeJ mice with AA were given either Ifidancitinib (n=5) or vehicle control (n=5) in chow diets for 12 wks. **(A)** Representative images of Ifidancitinib or control treated C3H/HeJ mice before or after 12 weeks treatment. **(B)** Percentage of skin hair loss or regrowth is shown before and after treatment. ***P < 0.001 (Unpaired Student t test). **(C)** Representative immunofluorescence images of skin sections stained with anti-CD8, anti-MHC-I, or anti-MHC-II mAbs. Dashed scale bars represent 100 μm. **(D)** and **(E)** Representative FACS plots of cell suspension of mouse skin and the frequencies of infiltrating CD45^{+} leukocytes, IFN-γ-producing CD8^{+}NKG2D^{+} T cells and T cell differentiation and activation markers in skin of Ifidancitinib treated mice and control mice. **P < 0.01, ***P < 0.001 (Unpaired Student t test).

properties as a potent JAK1/3 inhibitor, we postulated that in addition to inhibiting the generation and proliferation of alopecic T cells, Ifidancitinib might also may also selectively induce effector T cell exhaustion in treated mice. Indeed, we observed an increase in the percentage of CD44^{+} effector T cells coexpressing high levels of PD-1 and TIM-3 in the SDLNs and spleen in Ifidancitinib-treated mice compared to control mice (Figures 4A, B). These PD-1-expressing T cells also expressed high levels of Eomes that is known to be highly expressed in exhausted T cells co-expressing inhibitory receptors (Figures 4C, D) (26). We next examined cytokine secretion by PD-1^{+}TIM-3^{+}CD8^{+} T cells in response to stimulation. PD-1^{+}TIM-3^{+}CD8^{+} T cells from Ifidancitinib-treated mice displayed decreased production of IFN-γ compared to the T cells from control

mice (Figures 4E, F). Taken together, these findings are consistent with the functional exhaustion of effector CD8^{+} T cells associated with coexpression of PD-1 and TIM-3 and decreased IFN-γ production. Thus, the effect of Ifidancitinib on AA prevention and reversal may be due to the induction of T cell exhaustion, in addition to inhibitory effects on T cell proliferation, differentiation and survival.

γc cytokines regulated effector T cell exhaustion

To gain insight into the underlying mechanisms of the upregulated co-inhibitory receptor expression on T cells after
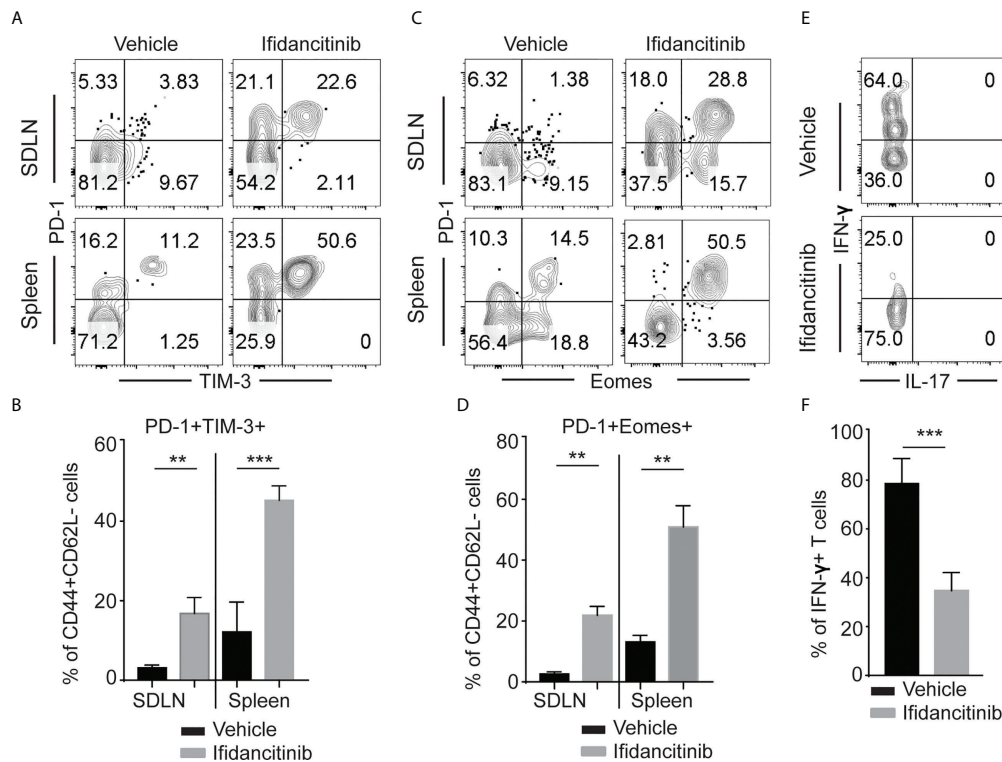
FIGURE 4

Ifidancitinib induced multiple co-inhibitory receptors in CD44$^+$CD62L$^-$ CD8$^+$ T cells. Mice were treated as in Figure 3. **(A)** and **(B)** The frequency of PD-1$^+$, Tim-3$^+$ and Eomes$^+$ T cells within CD44$^+$CD62L$^-$ CD8$^+$ T cells populations within the SDLNs of mice treated with Ifidancitinib or vehicle. **P < 0.01, ***P < 0.001. (Unpaired Student t test). **(C)** and **(D)** The frequency of PD-1$^+$, Tim-3$^+$ and Eomes$^+$ T cells within CD44$^+$CD62L$^-$CD8$^+$ T cells populations within spleen of mice treated with Ifidancitinib or vehicle. **P < 0.01. (Unpaired Student t test). **(E)** and **(F)** The frequency of IFN-γ producing cells within CD44$^+$PD-1$^+$Tim-3$^+$CD8$^+$ T cells from Ifidancitinib-treated mice or vehicle treated mice. ***P < 0.001. (Unpaired Student t test).

JAK1/3 inhibition, we stimulated T cells in the presence of Ifidancitinib or vehicle control *in vitro*. T cells increased expression of PD-1 after stimulation. Ifidancitinib treatment increased the expression levels of PD-1 on both CD4 T and CD8 T cells compared to vehicle control (Figures 5A, B and Figure S4A). Similar to Ifidancitinib, STAT5-IN-1 (STAT5 inhibitor) treatment also increased PD-1 expression on both CD4 T and CD8 T cells (Figures 5C, D and Figure S4B). Similarly, JAK1 or JAK3 inhibition by JAK1-selective inhibitor Itacitinib, JAK3-selective inhibitor Ritlecitinib, JAK1/2-selective inhibitor Ruxolitinib, or pan-JAK inhibitor Tofacitinib but not JAK2-selective inhibitor Fedratinib (27), increased PD-1 expression on both CD4 T and CD8 T cells (Figures 5E, F and Figure S4C), indicating that JAK1/3-STAT5 pathway might regulate PD-1 expression on activated T cells (28).

We next evaluated the potential contribution of endogenous γ chain cytokine produced by activated T cells to regulate PD-1 expression on activated T cells. Since activated T cells are the major source of IL-2, we simulated T cells with anti-CD3 and anti-CD28 in the presence of anti-IL-2 neutralizing mAbs. We observed that IL-2 neutralization increased PD-1 expression compare to Abs isotype control (Figures 5G, H and Figure S4D), indicating that endogenous IL-2 was critical for PD-1 downregulation *in vitro* (28).

Transcription factors thymocyte selection-associated HMG box (TOX) and EOMES have been shown to promote T cell exhaustion in cancer and chronic viral infections (26, 28–30). We measured the expression levels of TOX and EOMES in T cells after individual JAK-selective inhibitor treatment. We observed that JAK1 or JAK3 inhibition by selective inhibitors robustly increased the frequency of Eomes$^+$TOX$^+$ in both CD4 T and CD8 T cells T cells compared to vehicle and JAK2 inhibitor treatment *in vitro* (Figure 5I and Figure S4E). Similar to JAK1 or JAK3 inhibition by selective inhibitors, IL-2 neutralization promoted the generation of Eomes$^+$TOX$^+$ T cells *in vitro* (Figure 5J).

To evaluate the potential contribution of γ chain cytokine in regulating PD-1 expression and T cell exhaustion *in vivo*, we
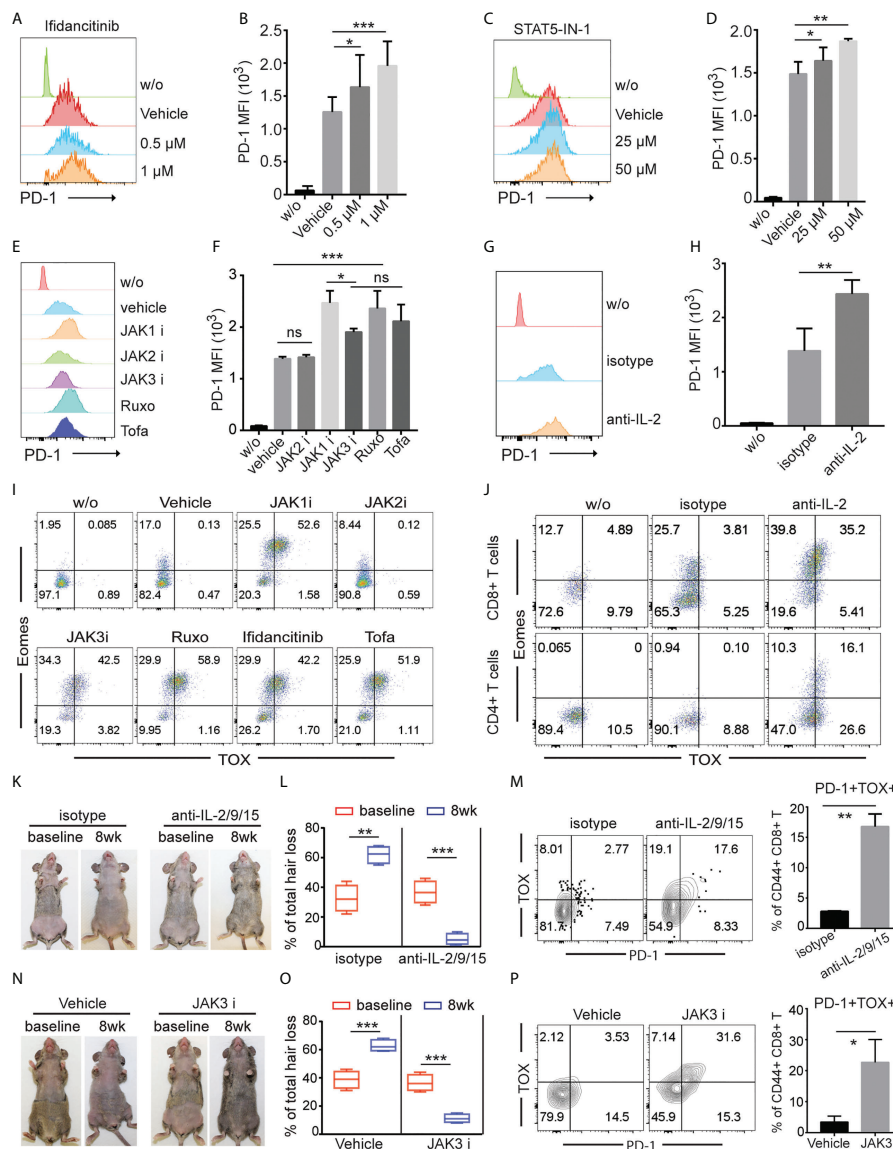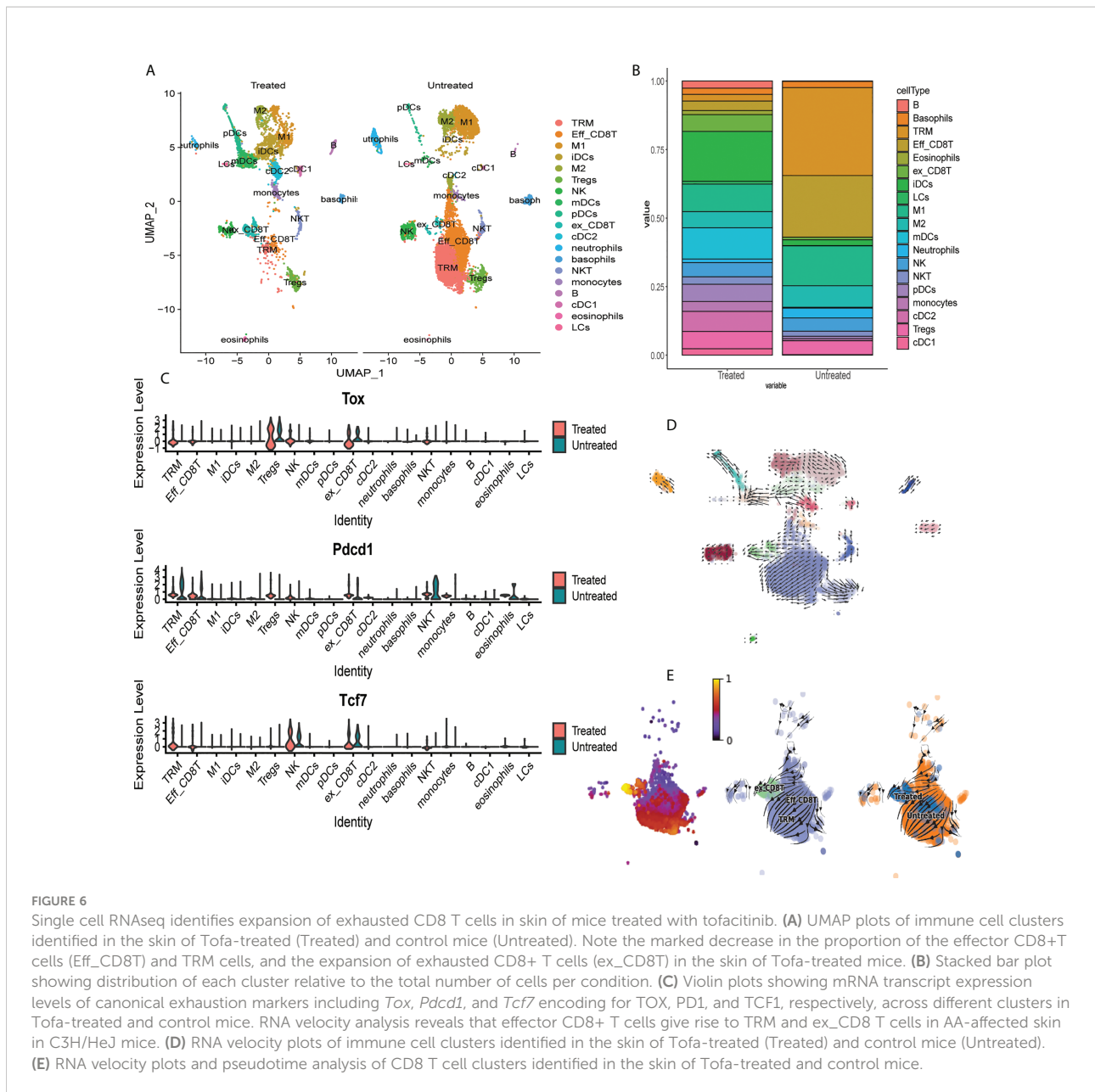
FIGURE 5

γc cytokines regulated effector T cell exhaustion. **(A)** to **(J)** T cells from C3H/HeJ mice without AA were stimulated with 500 ng/ml anti-CD3 in the presence of indicated regents *in vitro* for 4 (d) **(A)** and **(B)** The expression of PD-1 on CD8+ T cells was measured by FACS after treated with increasing dose of Ifidancitinib. *P < 0.05, ***P < 0.001 (one-way ANOVA). **(C)** and **(D)** The expression of PD-1 on CD8+ T cells was measured by FACS after treated with increasing dose of STAT5 inhibitor. *P < 0.05, ***P < 0.001 (one-way ANOVA). **(E)** and **(F)** The expression of PD-1 on CD8+ T cells was measured by FACS after treated with 1 μM of JAK1i (Itacitinib), JAK2i (Fedratinib), JAK3i (Ritlecitinib), JAK1/2-selective inhibitor Ruxolitinib (Ruxo), or pan-JAK inhibitor Tofacitinib (Tofa). ns indicates not significant, *P < 0.05, ***P < 0.001. P values were determined using one-way ANOVA followed by Brown-Forsythe test. **(G)** and **(H)** The expression of PD-1 on CD8+ T cells was measured by FACS after treated with 20 μg/ml IL-2 neutralizing mAbs. **P < 0.01. (Unpaired Student t test). **(I)** The expression of Eomes and TOX in CD8+ T cells was measured by FACS after treated with 1μM of JAK1i (Itacitinib), JAK2i (Fedratinib), JAK3i (Ritlecitinib), JAK1/2-selective inhibitor Ruxolitinib (Ruxo), Ifidancitinib, or pan-JAK inhibitor Tofacitinib (Tofa). **(J)** The expression of Eomes and TOX in CD8+ T cells was measured by FACS after treated with 20 μg/ml IL-2 neutralizing mAbs and isotype control mAbs. **(K)** to **(M)** C3H/HeJ mice with AA were treated with a combination of IL-2 neutralizing mAbs, IL-9 neutralizing mAbs and IL-15 neutralizing mAbs or isotype for 8 weeks. **(K)** Representative images of anti-IL2/9/15 or isotype treated C3H/HeJ mice before or after 8 weeks treatment. **(L)** Percentage of skin hair loss or regrowth is shown before and after treatment. **P < 0.01, ***P < 0.001 (Unpaired Student t test). The expression of PD-1 on CD8+ T cells was measured by FACS after treated with 20 μg/ml IL-2/9/15 neutralizing mAbs. **P < 0.01. (Unpaired Student t test). The results are representative of two separate experiments. **(N)** to **(P)** C3H/HeJ mice with AA were treated with Ritlecitinib or vehicle systemically for 8 weeks. **(N)** Representative images of Ritlecitinib or vehicle treated C3H/HeJ mice before or after 8 weeks treatment. **(O)** Percentage of skin hair loss or regrowth is shown before and after treatment. ***P < 0.001 (Unpaired Student t test). **(P)** The frequency of PD-1+TOX+CD44+CD8+ T cells within SDLNs were measured with mice that were systemically treated with Ritlecitinib or vehicle for 8 weeks. *P < 0.05. (Unpaired Student t test). The results are representative of two separate experiments. The results are representative of two separate experiments.

FIGURE 6

Single cell RNAseq identifies expansion of exhausted CD8 T cells in skin of mice treated with tofacitinib. **(A)** UMAP plots of immune cell clusters identified in the skin of Tofa-treated (Treated) and control mice (Untreated). Note the marked decrease in the proportion of the effector CD8+T cells (Eff_CD8T) and TRM cells, and the expansion of exhausted CD8+ T cells (ex_CD8T) in the skin of Tofa-treated mice. **(B)** Stacked bar plot showing distribution of each cluster relative to the total number of cells per condition. **(C)** Violin plots showing mRNA transcript expression levels of canonical exhaustion markers including *Tox*, *Pdcd1*, and *Tcf7* encoding for TOX, PD1, and TCF1, respectively, across different clusters in Tofa-treated and control mice. RNA velocity analysis reveals that effector CD8+ T cells give rise to TRM and ex_CD8 T cells in AA-affected skin in C3H/HeJ mice. **(D)** RNA velocity plots of immune cell clusters identified in the skin of Tofa-treated (Treated) and control mice (Untreated). **(E)** RNA velocity plots and pseudotime analysis of CD8 T cell clusters identified in the skin of Tofa-treated and control mice.

treated C3H/HeJ AA mice with a combination of IL-2 neutralizing mAbs, IL-9 neutralizing mAbs and IL-15 neutralizing mAbs (anti-IL-2/9/15). We observed increased frequency of PD-1+TOX+CD44+ CD8 T cells with anti-IL-2/9/15 treated mice compared to isotype control treated mice (Figures 5K–M). Further, JAK3-selective inhibitor Ritlecitinib increased frequency of PD-1+TOX+CD44+ CD8 T cells (Figures 5N–P). These results indicated that γc cytokine regulates T cell survival and exhaustion, and pharmacological inhibition of JAK1 or 3 signaling or neutralizing γc cytokine promote effector T cell exhaustion (23).

Single cell RNAseq identifies expansion of exhausted CD8 T cells in skin of mice treated with tofacitinib

To determine the effects of JAKi on induction of exhaustion phenotype in CD8+ T cells, we utilized scRNAseq to characterize T cell composition in AA skin after treatment with the pan-JAK inhibitor, Tofactinib (Tofa) (Figure S5). We analyzed single cell suspensions of pre-sorted CD45+ cells isolated from whole skin from C3H/HeJ untreated mice or mice treated with Tofa. 19 clusters representing diverse immune cell types have been identified by

scRNAseq (Figure 6A, Supplementary Table 1) (31). We identified a significant reduction in the proportion of TRM cells and Eff_CD8T cells (cluster 0 and cluster 1, respectively) in the skin of Tofa-treated mice, and a significant expansion in the proportion of ex_CD8T cells characterized by the expression of Tox (cluster 9) (Figures 6A–C). We used RNA velocity trajectory analysis to predict the origin trajectory of the ex_CD8T cell cluster (Figures 6D, E) (32). We identified that similar to TRM cells, ex_CD8T cells in the skin originate from the Eff_CD8T cell cluster (Figures 6D, E).

Discussion

The JAK/STAT pathway plays a critical role in regulating the immune system. As a result, targeting JAK/STAT pathways has shown great promise in the treatment of various autoimmune disorders, including AA (8, 9). We previously found that the lesional skin of AA-affected animals and humans display prominently activated IFN and γc cytokine pathways, both of which are mediated through JAKs (7). This invited trials to target the JAK/STAT pathway for AA treatment, through which we identified selective small molecule JAK inhibitors as a new class of drugs that showed remarkable efficacy in the treatment of AA (7, 12–14).

JAK-mediated γc cytokine signaling is a crucial regulator of lymphocyte development, homeostasis, and function. Specifically, γc cytokines bind to receptors with a unique γc cytokine receptor subunit; the common γc portion of these receptors associate with JAK3 in order to activate STATs for downstream signaling (16). Thus, JAK3 emerged as a potential therapeutic target (33–35), however, recent studies also implicated a role for JAK1 in the pathogenesis of inflammatory diseases (36). JAK1 functions in concert with JAK3 to mediate downstream γc signaling; furthermore, a number of key inflammatory cytokines, such as type I and type II IFN, might depend on JAK1-mediated signaling in several autoimmune disorders, including AA (34). As a result, dual JAK1/3 inhibition would target both the JAK1-dependent inflammatory cytokines as well as the JAK1/3-dependentiactivation of γc cytokine receptor signaling, while sparing JAK2 inhibition (8, 9).

Here, we demonstrated that the JAK1/3 inhibitor Ifidancitinib has potent anti-inflammatory effects both in vitro and in C3H/HeJ mice in vivo. We showed that JAK1 inhibition by Ifidancitinib suppressed IFN-γ signaling, demonstrated by the reduced expression of IFN-γ-dependent genes such as MHC class I, CXCL10, and CXCL11. IFN-γ is prominently expressed in AA lesions and is believed to contribute to the collapse of HF immune privilege by upregulating the expression of MHC class I in the HF (3, 4, 7). Thus, the preventative and therapeutic effects of Ifidancitinib on AA mice may be in part due to the restoration of HF immune privilege by inhibiting IFN-γ and other forms of JAK1-dependent pro-inflammatory signaling (3, 4, 7).

Furthermore, we found that Ifidancitinib has potent effects on pathogenic T cells that contribute to AA onset and progression. We observed that the in vitro differentiation of naïve CD8+ T cells to NKG2D+CD8+ T cells, which are critical to AA pathogenesis (7), was drastically inhibited by Ifidancitinib. We also observed that both the prevention and reversal of AA upon Ifidancitinib treatment were associated with the suppression and reduction of lymphocytes including CD3+ T cells, CD4+ T cells, CD8+ T cells, and NKG2D+CD8+ T cells as well as CD8+CD44+CD62L- effector memory T cells that are associated with AA (7). Thus, our results demonstrate that the JAK1/3 pathway is critical to the differentiation of NKG2D+CD8+ T cells in AA, and that selective inhibition of JAK1/3 is capable of both disease prevention and reversal on the phenotypic level as well as the cellular level.

Recently, efforts to treat inflammatory disease are focused on not only suppressing T cell proliferation and differentiation, but also rendering pathogenic T cells unresponsive to pro-inflammatory signaling (23, 24). One such method is to induce T cell exhaustion, which is characterized by the loss of effector function and the expression of multiple inhibitory receptors such as PD-1, TIM-3, and LAG-3, as well as changes in transcriptional signature (23). Previous studies showed that exhausted, unresponsive CD8+ T cells were associated with poor clearance of chronic viral infections as well as cancer cells, but were predictive of good prognosis in several autoimmune diseases (37). The induction of T cell exhaustion remains incompletely understood, however, it has been shown that persistent antigen stimulation, proinflammatory and suppressive cytokines, and regulatory leukocytes all play important roles in driving T cell exhaustion (23). Notably, high expression of PD-1 on HIV-specific exhausted cytotoxic T lymphocytes were associated with the disruption of the γc cytokine network in T cells during HIV infection (38). Since γc signaling is also involved in AA pathogenesis and mediated by the JAK/STAT pathway, we investigated whether Ifidancitinib is able to induce T cell exhaustion. Indeed, JAK1/3 inhibition by Ifidancitinib resulted in increased expression of PD-1 and TIM-3 on CD8+CD44+CD62L- effector memory T cells. Furthermore, these T cells exhibited reduced IFN-γ production, which is consistent with the loss of effector function observed in exhausted T cells (23, 24). Further, we found that γc cytokines regulated effector T cell exhaustion in vitro and in vivo through other JAK1 or JAK3 inhibition or by neutralizing γc cytokines. This is consistent with a recent report that STAT5 represses the expression of Tox, Pdcd1, Tim3, Lag3 (39). Reduced common γc cytokine-JAK1/3-STAT5 signaling achieved by JAK inhibitors might be one of the underlying molecular mechanisms that induce T-cell exhaustion.

In AA patients successfully treated with tofacitinib or ruxolitinib, a significant number of patients experience relapse upon discontinuation of treatment (12, 13, 40, 41). While this phenomenon has not been explored in the context of T cell exhaustion, inducing pathogenic T cells unresponsive to pro-inflammatory signaling events that re-establish disease onset may help prevent relapse recurrence (24). Further studies are needed to test the lasting effects of JAK inhibitor induced mediated T cell exhaustion on AA pathogenesis, but the

potent loss of effector T cell function induced by Ifidancitinib treatment is encouraging. Treatment regimens that maintain T cell exhaustion after acute treatments may prove to have long-term benefits (23, 24).

In summary, we demonstrate that inhibition of JAK1/3 signaling inhibits the differentiation and proliferation of cytotoxic NKG2D$^+$CD8$^+$ T cells, induces effector T cell exhaustion, and suppresses immune infiltrates in skin for both disease prevention and reversal.

Materials and methods

Mice and study approval

C3H/HeJ, C57BL/6 (B6), CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ) were purchased from The Jackson Laboratory and were maintained under specific pathogen-free conditions at the animal facility at Columbia University Medical Center (CUMC). All protocols were approved by the Institutional Animal Care and Use Committee of CUMC.

Generation of C3H/HeJ AA mice

Surgical methods for skin grafts to induce AA in C3H/HeJ mice were described previously (7). For the skin graft model, around 10 mm diameter skin from C3H/HeJ AA mouse was grafted to 10-week-old C3H/HeJ recipient and covered with bandage for 10 days. Hair status was examined twice weekly.

Antibodies and flow cytometry

Antibodies used in the experiments with mouse cells were obtained from Biolegend unless otherwise stated: anti-CD3 (17A2); anti-CD4 (GK1.5); anti-CD8 (53-6.7); anti-B220 (RA3-6B2); anti-CD25 (PC61.5); anti-CD44 (IM7); anti-CD45 (30-F11); anti-CD62L (MEL-14); anti-CD45.1(A20); anti-CD45.2 (104); anti-NKG2D (CX5); anti-PD-1 (29F.1A12); anti-TIM-3 (RMT3-23); anti-CD200 (OX-90); anti-EpCAM (G8.8); anti-MHC I (36-7-5); anti-MHC II (M5/114.15.2); anti-LAG-3 (C9B7W; Thermo Fisher Scientific); anti-pan NK cells (DX5; Thermo fisher); anti-Foxp3 (FJK-16s; Thermofisher); IL-17 (TC11-18H10.1); anti-TNF-α (TN3-19.12); anti-IFN-γ (XMG1.2); anti-Eomes (Dan11mag; Thermo fisher); anti-TOX (REA473; Miltenyi Biotec), anti-pStat1 (Tyr701) (58D6; Cell Signaling); anti-pStat5 (Tyr694) (C71E5; Cell Signaling); anti-Actin (SCBT). The following mAbs raised against human antigens were all purchased from Biolegend: CD3 (OKT3); CD4 (OKT4); CD8 (SK1); NKG2D (1D11); IL-17 (BL168); IFN-γ (B27). Viable cell populations were gated based on forward and side scatters and by fixable blue (Thermo Fisher

Scientific) staining. Samples were collected on an LSR II and analyzed with FlowJo software (Tree Star).

Cytokines and JAK inhibitor

Recombinant murine and human IL-2, IL-15 and IFN-γ were from Peprotech. Ifidancitinib chow diet and Ifidancitinib were provided by Rigel Pharmaceuticals and Aclaris Therapeutics. Itacitinib (catalog HY-16997, MedChemExpress), Fedratinib (catalog 202893, Medkoo), Ritlecitinib (catalog PZ0316, MilliporeSigma), Ruxolitinib (catalog S1378, Selleck), Tofacitinib (catalog 200811, Medkoo)

Mice treatment

Newly grafted C3H/HeJ mice or long standing C3H/HeJ AA mice were fed with Ifidancitinib incorporated chow diet (0.5 g/kg) for indicated time. Mice were scored weekly for signs of hair regrowth and loss. Mice were euthanized and organs were collected for analysis after treatment.

Ritlecitinib was delivered through an ALZET osmotic pump (MODEL 1002, DURECT). For topical treatment, C3H/HeJ AA mice were topically treated with 2% (w/w) Tofacitinib in Aquaphor (Aquaphor) twice daily. For antibody treatment, newly onset C3H/HeJ mice with AA were administered through IP injection with 0.1 mg combination of IL-2 neutralizing mAbs (JES6-1A12; Bioxcell), IL-9 neutralizing mAbs (9C1; Bioxcell) IL-15 neutralizing mAbs (AIO.3; Bioxcell) or Rat IgG2a (Bioxcell) twice weekly for 8 weeks.

Mouse tissue processing

Skin was finely minced and digested for 45 min at 37°C with 2 mg/ml collagenase type IV (Worthington) and 0.05 mg/ml DNase I (Sigma-Aldrich) in RPMI 1640. Digested skins were filtered through a 70 μm cell strainer. SDLNs or spleens were homogenized and filtered through a 70 μm cell strainer. Splenocytes were depleted of erythrocytes by RBC Lysis Buffer (Thermo Fisher Scientific). Cells were either re-suspended in FACS buffer (PBS with 2% FCS) for flow cytometric staining, stimulated with PMA and ionomycin for cytokine production or single-cell library construction.

In vitro mouse T cell stimulation, differentiation and cell proliferation assay *in vitro*

For NKG2D$^+$CD8$^+$ T cell differentiation, purified C3H/HeJ naïve CD8$^+$ T cells were stimulated with IL-15 (20 ng/ml) in the

presence of Ifidancitinib or vehicle control (DMSO) for 4 d. For *in vitro* proliferation, CellTrace Violet-labeled T cells were stimulated with plate-bound anti-CD3 Ab (0.5 μg/ml) and IL-2 (5 ng/ml), and cell proliferation was assessed by dye dilution. For PD-1 induction, purified C3H/HeJ naïve CD4^{+} T or CD8^{+} T cells were stimulated with 500 ng/ml anti-CD3 in the presence of indicated regents *in vitro* for 4 d described in Figure 5.

Adoptive transfer of T cell subsets and *in vivo* proliferation

Purified T cells from CD45.1-congenic B6 mice were stimulated with plate-bound anti-CD3 Ab (1 μg/ml) and IL-2 (5 ng/ml) *in vitro* for 3d. CellTrace Violet-labeled T cells were adoptively transferred i.v. to B6 (CD45.2) mice. After 12 h, mice were treated with various dosages of Ifidancitinib and 20 μg rhIL-2 as described previously. On day 3, mice were sacrificed and the proliferation of donor T cells from their spleens and SDLNs were analyzed by flow cytometry.

Intracellular stimulation

SDLN or skin single cell suspensions were cultured with 1× Cell Stimulation Cocktail (Thermo Fisher Scientific). After 1 h, 1× Protein Transport Inhibitors) (500X) (Thermo Fisher Scientific) was added, followed by additional 4 h incubation at 37° C. The cells were then fixed, and permeabilized using FoxP3 fixation/permeabilization kit (Thermo Fisher Scientific) and stained intracellularly with anti-IFN-γ, IL-4, IL-17 or TNF-α for 60 min at 4° C.

Stimulation of human T cells

Purified human CD8^{+} T cells were stimulated *in vitro* with IL-15 (20 ng/ml) in the presence of Ifidancitinib or vehicle control (DMSO) for 4 d. The frequency of NKG2D^{+}CD8^{+} T cell was assessed by flow cytometric analysis. For cell proliferation, CellTrace Violet-labeled T cells were stimulated with plate-bound anti-CD3 Ab (1 μg/ml) and IL-2 (10 ng/ml), and cell proliferation was assessed by dye dilution.

Immunofluorescence staining and western blot

Immunofluorescence staining of frozen skin sections was performed as previously described., The individual primary Ab was used at optimal concentrations for detection (final concentration 10ug/ml), followed fluorochrome-conjugated goat anti-rat secondary Abs (Thermo Fisher Scientific). The

immunofluorescence staining of mouse MHC class I was performed using biotin labeled mouse anti-H-2Kk, followed by fluorochrome-conjugated streptavidin (Thermo Fisher Scientific). Murine T cell blasts were pretreated in the absence or presence of Ifidancitinib (0.1, 0.3 and 1 μM) for 1 h and then stimulated with IL-2 (20 ug/ml) or IFN-γ (40 ug/ml) for 15 min. Total cell lysate (40 ug) was separated by electrophoresis and probed with Abs as described previously.

Mouse skin bulk RNA sequencing and bioinformation analysis

Total cellular RNA was extracted using RNeasy Kit (Qiagen) from skin homogenates. RNA quality and quantity were determined using an Agilent BioAnalyzer (Agilent Technologies). RNA isolation, library construction and sequencing were performed at GENEWIZ. Gene-expression analysis further was performed with the Seurat R package (27).

Single-cell library construction

To prepare mouse skin single-cell suspensions of immune cells, live CD45+ cells were FACS sorted. The samples were submitted to the JP Sulzberger Columbia Genome Center's Single Cell Analysis Core. There, the Chromium Next GEM Single Cell 5' Reagent Kit V2 was used to prepare cell libraries according to the manufacturer's instructions, with a target of 5000 cells and 350 million reads. Libraries were sequenced on an Illumina NextSeq 500/550.

Bioinformatics analysis

After sequencing, FASTQ files were processed using the CellRanger v6.1.2 and aligned to the mm10-2020-A reference transcriptome. The output CellRanger files of the individual samples were merged into a single Seurat object and analyzed using Seurat package v4.0.6 in R v4.1.0 (31). For quality control, we removed cells with lower than 500 molecules detected within a cell and cells with more than 5% of UMIs were derived from mitochondrial genes, and included cells which had more than 200 genes but less than 3000 genes. Post quality control, 7127 cells for untreated skin and 4230 cells for Tofa-treated skin remained for downstream bioinformatics analyses. We then used Seurat package to integrate the individual samples, perform a global-scaling normalization and linear transformation using 'NormalizeData', and 'ScaleData' functions, respectively. We next used the 'Elbowplot' function, to determine the dimensionality of the dataset, and subsequently used 30 PCAs to cluster the cells using the Louvain algorithm. We then performed uniform manifold approximation and projection

(UMAP) dimensionality reduction to explore and visualize the dataset. We used 'FindAllMarkers' function in Seurat to find differentially expressed markers for each cluster and used canonical markers to match the unbiased clustering to known cell types as outlined in Supplementary Table 1. We used 'VlnPlot' and 'FeaturePlot' functions in Seurat to show expression probability distributions across clusters and visualize feature expression on a PCA plot.

To perform RNA velocity analysis in our scRNASeq dataset, we used the Python v.3.11 based package scVelo, which uses normalized Seurat object in conjunction with RNA Velocity analysis (32). Briefly, we used Samtools v1.10 and Velocyto to generate Loom files for the individual single cell samples we analyzed using Seurat. Then, we integrated the individual loom files and the Seurat meta-data using 'anndata' function. Then, we merged the individual samples into one anndata object and added UMAP coordinates from the merged Seurat object to match the order of the Cell IDs in the anndata object. At last, we used scVelo to generate RNA Velocity plot based on Seurat UMAP coordinates and the 'subset' function to generate RNA Velocity plot for the CD8 T cell subsets (cluster 0, 1, and 9).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 7.0 software. Data were expressed as mean plus or minus SEM for each group and results were compared with 2-tailed t tests, log-rank test, or one-way ANOVA. n.s.> 0.05, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive (SRA), accession number PRJNA863610.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of CUMC.

Author contributions

ZD and AC conceived the study. ZD, TS, YC, and EL performed the experiments. TS, YC, EL, and EW analyzed data and provided critical review of the manuscript. ZD and AC analyzed data wrote the manuscript. AC supervised the study and provided funding. All authors contributed to the article and approved the submitted version.

Conflict of interest

Columbia University has licensed patents on the use of JAK inhibitors in alopecia areata to Aclaris Therapeutics, Inc. AC is a consultant to Almirall, Janssen Arcturus and a shareholder of Aclaris Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.955038/full#supplementary-material

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